Comparative analysis of gut microbes in acute anaemic mice model treated with iron nanoparticles synthesised using *Colocassia esculenta* leaf extract.

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

Submitted By
SRIJEETA GHOSH
Registration ID:230020368978
Enrolment ID:2322210002036

Advisor
Dr. Mausumi Sikdar(ne'e Bhakta)
Designation- Professor
Affiliation

School of Health Science and Translational Research
Sister Nivedita University ,Kolkata
June 2025

SNU
SISTER NIVEDITA UNIVERSITY

SNU IQAC

DECLARATION BY THE STUDENT

ıereby declare that the work which is being presented in this dissertation entitled "Comparative analysis of gut ıicrobes in acute anaemic mice model treated with iron nanoparticles synthesised using *Colocassia sculenta* leaf extract." in partial fulfilment of the requirements for the award of the degree of achelors/**Masters**/Ph.D. of Srijeeta Ghosh in Sister Nivedita University is an authentic record of my own work ırried out during the period from January,2025 to May 2025 under the supervision of Dr Mausumi Sikdar(nee hakta)Department of Life Science, Presidency University.
acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and revention of Plagiarism in Higher Educational Institutions*).

confirm that this dissertation has been checked for plagiarism using the plagiarism checking software **Turnitin.** At e end of the dissertation, a copy of the summary report demonstrating similarities in content and its potential ource (if any), generated online using plagiarism checking software is enclosed. I herewith confirm that the ssertation has been less than 10% similarity according to the plagiarism checking software's report and meets the GC Regulations as well as plagiarism rules of Sister Nivedita University.

urther declare that no portion of the dissertation or its data will be published without the Institute's or supervisor's rmission. I have not previously applied for any other degree or award using the topics and findings described in y dissertations.

Srijeeta Ghosh 28/11/25

gnature of the students with Date)

me of the Student- Srijeeta Ghosh

gistration no.-230020368978

partment-Biotechnology

Galaxy S23

PRESIDENCY UNIVERSITY
KOLKATA

Presidency University

Hindoo College (1817-1855), Presidency College (1855 – 2010)

Date: 04/06/2025

CERTIFICATE

This is to certify that the M.Sc. dissertation thesis entitled **'Comparative analysis of gut microbes in acute anaemic mice model treated with iron nanoparticles synthesised using *Colocassia esculenta* leaf extract '** is submitted by **Ms. Srijeeta Ghosh**, Registration ID:230020368978 & Enrolment ID:2322210002036 of M.Sc. 4th Semester of the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata. The work leading to this thesis was done by her during the period, January, 2025 to May 2025, for the partial fulfillment of her Master's degree in Biotechnology. She has worked independently under my guidance and completed it to my satisfaction.

Sikdar 04/06/2025

Dr. Mausumi Sikdar (ne'e Bhakta),
Professor in Physiology,
Department of Life Sciences,
Presidency University,
Kolkata.

Dr. Mausumi Sikdar (ne'e Bhakta)
Professor
Department of Life Sciences
Presidency University
Kolkata -700 073

86/1, College Street, Kolkata - 700 073, Phone : (033) 2241 1960, Fax : (033) 2241 2738

Galaxy S23

SISTER NIVEDITA UNIVERSITY
সিস্টার নিবেদিতা বিশ্ববিদ্যালয়
DG Block - 1/2, Action Area I, New Town, Kolkata - 700156, West Bengal, India.

DEPARTMENT OF BIOTECHNOLOGY & DEPARTMENT OF MICROBIOLOGY
জৈব প্রযুক্তি বিভাগ ও অণুজীব বিদ্যা বিভাগ

TO WHOM IT MAY CONCERN

This is to certify that the project report titled "_Comparative analysis of gut microbes in acute anaemic mice model treated with iron nanoparticles synthesised using *Colocassia esculenta* leaf extract.", submitted by Ms.Srijeeta Ghosh (Registration No-230020368978 Enrolment No-2322210002036) to the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by him/her for the partial fulfillment of the requirements for the Master of Science degree.

Date: 03.06.2025.

Place: SISTER NIVEDITA UNIVERSITY

Signature of Head of the Department

Seal

Head
Department of Biotechnology
Sister Nivedita University
Block DG-1/2, Action Area 1
New Town, Kolkata-700 156

Acknowledgement

I am profoundly grateful for the incredible opportunity to undertake my dissertation at the esteemed Presidency University, Kolkata. This journey has been nothing short of transformative, providing me with deep insights, invaluable experiences, and the opportunity to engage with some of the brightest minds in my field .I owe my deepest gratitude to Prof. Mausumi Sikdar (ne'e Bhakta), whose mentorship has been a beacon of inspiration and guidance. Her profound expertise and unwavering dedication to advancing knowledge have left an incredible mark on my academic journey. Prof. Mausumi Sikdar (ne'e Bhakta) encouragement to explore beyond conventional boundaries and her commitment to fostering critical thinking have helped me grow immensely. Her supportive guidance has been a privilege and honour, and her approach to research and ship has inspired me to aspire to greater academic and professional heights. I am also sincerely thankful to Prerona Biswas, Sambit Mandi, Rabindar Sha, Sudipta Majhi, Nandini Roy, Ananya Basu whose patience, insights, and camaraderie provided a strong foundation during my internship. Their openness and willingness to assist me throughout my research made the learning process smoother and more enjoyable. The scholar's genuine warmth, encouragement, and collaborative spirit have been deeply motivating, and their support has been invaluable in overcoming challenges and navigating new academic territories. This internship has been a milestone in my career, enabling me to acquire new skills and refine my understanding in ways I had only dreamed of. I am indebted to Prof. Mausumi Sikdar (ne'e Bhakta), and the scholars for their unwavering support, and I leave this experience filled with gratitude and renewed dedication to my studies.

ABBREVIATIONS

1. CENP- Colocassia Esculenta Nanoparticle.
2. PHZ- Phenyl Hydrazine.
3. INP- Iron nanoparticle
4. MR- Methyl Red.
5. VP- Vogues-Proskauer
6. TSI- Triple Sugar Iron
7. PBS- Phosphate Buffer Saline
8. CV- Crystal Violet

ABSTRACT

In recent decades the gut microflora has incited interest among various research groups due its myriad application in modulating the body homeostasis. The diverse bacteria present in gut microflora elicits interest among researchers, gut microbiota which is a diverse area consisting of various microorganisms both aerobic and anaerobic, with the latter being predominant. The homeostatic behaviour of the gut microbiome enables it to have an active role in overall health, immunity and metabolism. The composition of the gut microbiome is affected by different environmental and physiological factors. Interestingly, recent literature predicates the alteration of in gut microflora among diseased and healthy individuals. Here, our study encompasses the comparative analysis of the gut microflora in anaemia, anaemia treated with iron nanoparticles (CeNP) with normal female Swiss albino mice. In our study we have induced anaemia in 4 weeks old female mice with phenyl hydrazine with a dose of 20 mg/kg body weight. The mice were intravenously injected with phenyl hydrazine three times with an injection on day 1,3 and 5th day. Subsequently, the mice were treated with biosynthesized iron nanoparticles with intraperitoneal injection for 15 consecutive days. The haemoglobin level, haematocrit percentage, RBC cell count was done on days 1,3,5 and also on day 7, the day of sacrifice. Post treatment the mice were euthanised and the gut which is ileum ,colon, and faeces were collected. The samples are homogenized and isolated and divided into various groups. They are: anaemic, anaemia treated with nanoparticles, and only nanoparticles on healthy mice. Post sacrifice the bacteria were isolated from the organs using spread plate technique on nutrient agar plates. Pure colonies were isolated by streaking single colonies in a nutrient agar plate. The characterization assessment :Gram staining and various biochemical methods were performed to characterize the different kinds of bacteria found in different groups .IMViC ,Urease and TSI test is performed to further characterize the type of bacteria found. Methyl red (MR) positive test was observed in the respective groups which indicate mixed acid fermentation tests were selectively positive, particularly in FeNP-only groups, indicating acetoin production, and consequently, *Enterobacter spp.* Citrate utilization and urease activity were also higher in nanoparticle exposed groups, indicative of what may be construed as a more original lifestyle probably induced by improved iron availability. (Triple Sugar Iron) Tests-The slant and butt color change together showed glucose and lactose/sucrose fermentation. For the most part, anaemic clones showed a reduced capacity for sugar utilisation. Catalase-positive strains were numerous in FeNP and control groups, whereas coagulase-positive strains were less common and were mainly observed in anaemic mice, indicating that possible 'opportunistic' pathogens including S. aureus were present during the disease. This test has shown a marked dissimilarity in antibiotic resistance profile: Anaemic mice strains showed broad resistance,(which were ampicillin and methicillin, respectively), suggesting higher susceptibility to multi-drug-resistant infections when challenged in an iron-starved state. The FeNP-induced groups were moderately to highly sensitive, predominantly to tetracycline and chloramphenicol (inhibiting zones ranged from 1.3 to 3.3 cm).Bacteria from the FeNP-only group exhibited lower resistance, suggesting that the microbiome was benefiting from a low pathogenic load. Vancomycin was moderately effective in all groups. The histopathological pictures were taken that indicated the difference in the groups. Antibiotic susceptibility tests were done to see the zone of inhibition of particular antibiotics. Various assays were done like DPPH and siderophore assay to understand the antioxidant and siderophore forming ability of the microbes respectively. Biofilm assay was done to understand the ability of the microbes for their biofilm production.

Keywords- Gut microbes, Iron deficiency anaemia, nanoparticle.

CONTENTS

SERIAL NO	TOPIC	PAGE NO
1	INTRODUCTION	8
2	REVIEW OF LITERATURE	10
3	MATERIALS AND METHODS	12
4	RESULT AND DISCUSSION	23
5	CONCLUSION	48
6	FUTURE PROSPECTS	50
7	APPENDIX	51
8	LIST OF FIGURES	54
9	LIST OF TABLES	55
10	REFERENCES	56

1. INTRODUCTION

Iron deficiency anaemia is a major health concern and it is vicariously spreading worldwide causing deaths and major health concerns for both males and females. However, the female population is more susceptible to anaemia due to menstruation and pregnancies.(1) But the risk of anaemia in males is increasing and the major factor of it is the age.(2) The effect of age is seen prevalent in males for anaemia than in females(3). A study conducted in a rural area of Haryana, India, found that the age-adjusted prevalence of anaemia among adult males was 27.9%. The study revealed a positive association between age and anaemia prevalence. Men aged 50–59 years had an odds ratio (OR) of 2.7 for anaemia compared to those aged 18–24 years. Men aged 60 years and above had an OR of 3.6 for anaemia compared to the 18–24 age group(4)

Iron deficiency anaemia is of various types: complete anaemia where there is a physiological loss in the blood count, and iron intake gets reduced in the body (5).The other type is when the iron incorporation in the body is reduced despites having storage for that (6). The other type is when the iron incorporation in the body is reduced despite having iron storage (7).

In recent decades the amalgamation of biotechnology with nanotechnology has elicited new horizons of research in the form of nanoparticles. Nanoparticles since their discovery have become an invaluable asset due to their myriad applications. Nanoparticles derived from different metallic particles such as copper, silver and zinc serve as very potent antimicrobial agents. Recent emergence of several antibiotic strains have helped nanoparticles gain substantial recognition against combating antibiotic resistance.(8–23) Similarly, the use of gold nanoparticles is widely known as a transfection agent where the suitable fragment of DNA is transported inside a cell(24–27). However, the development of iron nanoparticles have revolutionized the biotechnology field because of its multifaceted nature. Iron nanoparticles (INP) have various applications ranging from bactericidal agent, anti-cancer and antitumor agents, their photocatalytic activity can be used in bioremediation. In the field of biomedicine INP's can also be used as MRI contrasting agents. Thus, demonstrating our dependence on nanoparticles.(28–36)

However, since its development researchers have faced significant problems regarding the biocompatibility of the developed nanoparticles. Among the various techniques to develop nanoparticles, chemical synthesis is a method widely used around the globe. In chemical synthesis several chemical stabilizers are added in the reaction but as a result the developed nanoparticles often demonstrate acute toxicities and the chemical byproducts act as environmental pollutants.(37–41) The introduction of green technology aims to mitigate the toxicities caused by the chemical synthesis method. In green synthesis different plant parts are being used to extract phytochemicals which act as stabilizers during nanoparticles which substantially reduces the toxicities thus bringing out several translational applications(42–50).

In our study we have developed an anaemia model in female *Swiss albino* mice by injecting phenyl hydrazine which is known to destroy mature reticulocytes by inducing oxidative stress. Phenyl hydrazine acts by forming reactive oxidant species in RBCs which leads to oxidation of haemoglobin to methaemoglobin .These are further broken down into menichromes and Heinz bodies. This damage degrade the cytoskeletal proteins such as spectrin and initiate lipid peroxidation which leads to premature destruction of the erythrocytes. Macrophages clear the damaged erythrocytes which leads to loss in RBC count and haemoglobin concentration.(7,51)

Figure 2. Challenges and key points in reproducible synthesis of nanoparticles.

A microbiota is defined as a community of microorganisms that consists of various bacteria, archaea and other organisms that stay in specific environments. The human body is considered an environment for the bacteria to grow and survive. They are useful in many ways like detoxification reactions, immune system development and many more. The genomes are very dynamic and can vary based on different food, injection of foreign particles, age and others. Gut microbiota mainly colonize the gastrointestinal tract. Through literature study we got to know that iron nanoparticles are responsible for increase in *Firmicutes*, *Romboutsia,* and *Bilophila* and the *Bifidobacterium* abundance has decreased**.** Some studies showed that IoNP are non-toxic and have no significant changes in the gut microbiota but promote beneficial microbes. Previously said IONP can increase ROS production that have an effect on commensal microbes like *E.coli*, *Staphylococcus aureus* etc. However, some studies indicate that there are no changes in the gut microbes at all. All this made us question the topic and know more about it.(34-37).

Biofilms provide a niche for bacteria to survive in the harsh gut environment, resist the mechanical shear and escape from the immune monitoring. They reside in the mucosal surfaces, inside mucus layers, and on unprocessed food particles, all of which provide distinct microhabitats that bear specific metabolic traits. Biofilm associated bacteria are known to exist in a metabolically different state with respect to planktonic organisms.

They are able to ferment complex polysaccharides well, and are a dominant source of short chain fatty acids (SCFAs), including acetate and butyrate that are vital for colonocyte energy, and gut health overall. Biofilm bacteria are more resistant to antibiotics and adverse environments In bacteria; iron is an essential nutrient in that iron bioavailability may affect biofilm development and structure. Dysregulated iron levels could result in virulence factor expression also in commensal GI bacteria. This results in the conversion of commensals to pathobionts, which can be exacerbated in inflammatory or disease states including IBD and colorectal cancer. Correction of imbalanced iron status could inhibit or reverse pathogen-associated biofilm modifications and correct gut microbial homeostasis.(52–54).

2. REVIEW OF LITERATURE

The core digestive tract of the mice is studied intensively because of the striking similarities with the human gut. Correlation between GI tract and diseases can also be studied. Various symbiotic and commensal microorganisms are present in the gut (55–57).The microbiota that resides in the gastrointestinal tract forms a complicated ecosystem. They are essential physiological homeostatic in the host. Throughout the years ,sequencing has been levelled up and by that we can understand the gut flora composition and various genera of microbes. Most of the samples taken are usually from feces but ileum and colon are also a prime spot for gut microbiota isolation(1,58–64).There are many commensal, symbiotic and pathogenic bacteria that are present in the gut that survived the harsh intestinal physiochemical conditions. They actively involve with the host and live in the host's body. There may be various selection mechanisms and different microbe protective strategies to survive in the host's body(65–68).The variety of the microbiota was rich in colonic samples than in ileum samples. The dominant group in the ileum was Firmicutes. The abundance of Actinobacteria and Bacteroides was high in the colon region.*Lactobacilus* and *Turicibacter were* the predominates in the rat digestive tract. Some Firmicutes subgroups like Lachnospirachae and Ruminococcaceae were present in the colon.

Iron deficiency anaemia ,simply put, is a condition where the haemoglobin level decreases than the normal. It will cause various side effects like change in the gut microbiome composition and histopathological changes of the gut. It is predominantly seen in women (about 70%) and young children (40 percent). The primary goal of WHO was to reduce Anemia by 50% by the year 2025. One of the most basic micronutrients is iron which is helpful in numerous biochemical reactions. Haemoglobin is also of value as the enzyme components or otherwise in oxygen and for transport by other proteins implicated. Energy metabolism is the biological process, and cell proliferation are the locations where iron is required (1,62,69–71). Heme Iron, although absorbed more easily, exists in lesser extent in the body. An overload of iron results in oxidative stress and damage mediated by the reactive oxygen species. It can cause cell death. "Because iron naturally exists in its oxidized and insoluble form (as Fe3+), the reduced form (Fe2+) can be bound to protein such as heme for transport. The evidence from this transport by DMT1 suggests that nonheme iron is taken up as Fe2+. Duodenal cytochrome B is responsible for the reduction of iron. The primary function of the duodenum cytochrome B is iron absorption. Microbiota and iron are in a very complex relationship with each other. Supplementation of iron is detrimental for the gut microbiota (72).The excess iron causes a change in the intestine(73).Pathogenic strains of *E.coli* and *Salmonella* have siderophores that are useful for bacteria to survive in iron less environments. *Lactobacillus* and *Bifidobacterium* can grow without much iron portion(74–76). Literature review showed that foreign iron introduction could lead to reduction of beneficial microbes and increase of irrational microbes.

Table 1. Impact of oral iron supplementation on gut microbiota composition (↓—decrease, ↑—increase).

Bacteria	Iron Supplementation
Phylum: Firmicutes	↓
Genus: Enterococcus	↑
Genus: Lactobacillus	↓
Genus: Roseburia	↑
Genus: Clostridium	↑
Phylum: Proteobacteria	↑
Family: Enterobacteriaceae	↑
Species: E. coli	↑
Genus: Salmonella	↑
Genus: Shigella	↑
Genus: Citrobacter	↑
Order: Bacteroidales	↑
Genus: Bacteroides	↑
Genus: Campylobacter	↑
Genus: Bifidobacterium	↓
Genus: Prevotella	↓
Genus: Rothia	↓

Table 1. Impact of oral iron supplementation on gut microbiota composition (decrease and increase)

However, in rat tests, other researchers discovered an entirely unique result: oral iron supplementation enhanced the metabolic activity of the gut microbiota, as shown by the increased concentration, and promoted commensal and dominating bacteria in the colon of favourable short-chain fatty acids (65,75).Patients with iron deficiency have more bacteria including *Farcalibacterium prausnitzii*,*Ruminococcos bromii* etc. If we compare it with the control group the Bacteroidota, Patescibacteriaia, Actinobactota and Desulfobacterota abundance in the anaemic model group decreased, while the Firmicutes abundance got increased. Some experiments through literature study if founded out that abundance of *Lactobacillus* is prevalent in the anaemia model group than that is found in control group. A decrease in abundance of short chain fatty acid producing genera like *Roseburia*,*Butyricicoccus* are found in infant that is suffering from iron deficiency anaemia. A higher percentage of the variation in microbiota is found in the groups in large intestine as compared to small intestine.

Probiotics play a useful role in the gut. It is mostly used for maintaining the good microbes and inhabit the harmful ones. Some microbes are readily used as probiotics as they produce lactic acid when they undergo the process of fermentation. The most common example is *Lactobacillus*.(77,78).

3. MATERIALS AND METHODS

1. Preparation of the anaemic mice model
   a. *Swiss albino mice* were brought to the Central Animal Facility at Presidency Animal house. They were kept in a quarantine room for seven days and were provided with a standard lab diet and unrestricted access to water.
   b. Phenyl Hydrazine (PhZ) -98%(980ug/ul dilute 1:100=9.8ug/ul)was given to the mice at days 1,3,5.
   c. Nanoparticle concentration-1ug/ul after day 5 Phenyl hydrazine administration for 15 days.
   d. The mice were divided into groups of 4: healthy control mice, only anaemic mice (only PhZ was given),anaemic+nanoparticle(PhZ+NP),and only NP were the groups.
   e. The mice were euthanized, and the gut(ileum and colon) and feces was isolated in a 1.5ml microcentrifuge tube (TARSONS, India).
2. Preparation of *Colocasia esculenta* iron nanoparticles
   a) At first, fresh *Colocasia esculenta* leaves were collected and dried perfectly, making them in its powder form.
   b) Extraction was carried out by dissolving a certain amount of (5 g) *C. esculenta* leaf powder in 50 ml of absolute alcohol solution, and kept it undisturbed for 48 hr. Then the herbal extraction was collected, sealed, and stored at 4ºC for further use.
   c) The mixture was prepared by adding 10 ml of ethanolic solution of the extract to 50 ml of 100 mM $FeCl_3$.
   d) Within 1 hr., 1 ml of ethanolic extract was added after every 5 mins. A color change has shown from reddish brown to deep maroon green.
   e) 1 (N) NaOH solution was added dropwise and was checked with pH paper until the pH color changed, and came to pH 7.
   f) The OD of the precipitated solution was determined by a spectrophotometer. The maximum wavelength appeared at 192 nm.
   g) The solution was centrifuged (Model name-Spin Win) at 20ºC, 7500 rpm for 10 mins. The precipitate iron nanoparticles were dried in a concentrator, and made powder form with the help of a micro-pestle.

Isolation of single colonies of faecal and gut sample

FOR SERIAL DILUTION AND SPREAD PLATE.

- Samples from the mice
- Eppendorf tubes(TARSON)
- Test tube rack
- 1000μl micropipette
- Distilled water

FOR SPREAD PLATE

- Fresh Nutrient agar plates
- Erlenmeyer flask
- Nutrient agar
- Spreader · Inoculation loop · Ethanol · Spirit lamp

METHOD FOR SERIAL DILUTION AND SPREAD PLATE

- The isolated gut was placed in a 1X PBS buffer(pH-7.4) to remove surface debris and blood clots. It was then transferred to a 1.5 ml Eppendorf tube(TARSON) containing PBS buffer for homogenization using a micro pestle.
- The pellet was discarded, and the supernatant was transferred to a tube containing 1X PBS buffer.
- Faecal samples were collected from the lower colon of the large intestine into a 1.5 ml Eppendorf (TARSON) tube
- Serial dilutions were performed by transferring 100 µl of the supernatant PBS buffer to 900 µl of PBS buffer. This procedure was repeated to create dilutions from 10^1 to 10^6.
- Selected dilution(10^3) was spread on fresh Nutrient Agar plates and incubated overnight at 37◦C.
- The plates with different dilution were examined the following day and colonies of different morphologies were observed.
- Selected colonies were streaked on fresh Nutrient Agar plates to obtain pure colonies.
- Same procedure was performed for the isolation of single colonies of ileum, colon and faeces sample.

Constituents(HIMEDIA)	Standard value (g/l)	For media prepared (g/l)
Nutrient Agar	28	6.3
Distilled water	1000	225

Thus, 225ml of distilled water needs 6.3g of agar.

- In a conical flask the calculated amount of nutrient agar powder is added to distilled water
- The mixture is stirred thoroughly
- The flask is covered with cotton plug and is sterilized in the autoclave at 121◦C at 15 psi for 15 minutes.
- The sterilized agar is poured into a sterile petri dish in a sterile environment.

3.BIOCHEMICAL TESTS PERFORMED

Strains used for control

- *S.aureus*- ATCC 25923
- *Enterobacter*- 16s sequenced – PV330710
- *E.coli DH5α*

a. **Gram staining**:
It is done to see the types of bacteria found in each of the strains
MATERIALS REQUIRED
· Grease- free glass slides · Sterile inoculation loop · Spirit lamp REAGENTS REQUIRED · Crystal Violet, the primary stain · Iodine, the mordant · A decolourizer made of ethanol (95%) · Safranin, the counterstain.

Procedure of Gram Staining

1. A clean, grease free slide was taken.
2 Made a smear of suspension on that clean slide using loopful of sample
3. The smear is made of the strains.
4. Air dry and heat fix the slides.
5. Crystal Violet was added and left on the slides for 30 sec to 1 min, and then washed with distilled water.
6. Flood with the gram's iodine (for 1 min) and rinse using DI H2O.
7. Then rinse with 95% ethanol for 10sec-20sec and then water.
8. Add safranin for about 1 minute and wash with water.
9. Air dry, blot dry and Observe under Microscope(10X,40X and 100X).

IMViC TESTS

OBJECTIVE

The main objective of doing these biochemical tests is to make sure we understand the characteristic of the strains and compare between the groups.

MATERIALS REQUIRED · Sterile test tubes · Erlenmeyer flasks · Inoculation loop · Spirit lamp

INDOLE TEST

The indole test is a part of biochemical test that is used to see whether the microorganism can utilize tryptophan and produce indole.

PROCEDURE · Inoculate a tube of tryptone broth with a small quantity of a pure culture

PREPARATION OF TRYPTONE BROTH (100 ml)(HIMEDIA)

Tryptone broth	1 g
Sodium chloride	0.5g
Distilled water	100ml

Procedure

● Inoculation - Using a sterile inoculating loop, a small amount of the bacterial culture was picked and inoculated into a test tube containing tryptone broth. *E.coli* and *Enterobacter* control is taken as well

● Incubation - Incubate the inoculated broth at 35-37°C for 24-48 hours.

●Kovac's Reagent Addition- The broth after incubation was added with 0.5 mL of Kovac's reagent. Kovac's reagent that reacts with indole.

● Observation - Gently swirl the tube to mix.

· A positive indole test is detected by the development of a pink to red color (the "cherry-red ring") in the reagent layer above the medium within seconds of addition of the reagent

· If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.

This test is commonly used to differentiate between species, particularly among *Enterobacteriaceae.*

METHYL RED TEST

The methyl red test is a qualitative test used to determine the acidity of a culture medium, indicating whether an organism performs mixed acid fermentation.

Constituents(HIMEDIA)	Standard value g/l	For experiment g/ml
Dextrose	5	1.498
Dipotassium phosphate	5	1.07
Sodium chloride	1.4	1.07
Disodium phosphate	1.96	0.749
Monopotassium phosphate	1.5	0.749
Peptone	10	2.14
Distilled water	1000	214

Procedure

● Inoculation - Using a sterile inoculating loop, a small amount of the bacterial culture was transferred into a test tube containing MR-VP broth.

● Incubation - The inoculated broth is incubated at 35-37°C for 48 hours to allow fermentation to occur.

● Methyl Red Test - After incubation, 2-3 drops of methyl red indicator was added to the tube. Methyl red is a pH indicator; below pH 4.4 it is red and above pH 6.3 it is yellow.

● Observation- Tube was slightly shaken to mix the ingredients. The change in colour was noticed.

● Interpretation - Positive Result: If the broth turns red, it means the pH is of 4.4 or lower, which suggests that the organism has performed mixed acid fermentation and produced stable acids

Result- If the broth remains yellow (pH 6.2 or higher), it indicates that the organism did not produce significant acid during fermentation.

VOGES PROSKAUER TEST:

Mainly used for differentiation for *Enterobacter sp.*

· Barritt's reagent A: 5% (wt./vol) a-naphthol in absolute ethanol

· Barritt's reagent B: 40% (wt./vol) KOH in deionized water (this might be replaced by a 40% (wt./vol) NaOH solution)

· Reagents must be prepared fresh. Reagents are also referred to as VP1 and VP-2 or VP-A and VP-B.

Other materials –

· Sterile culture tubes

· Test and control bacterial strains (commonly used controls are *Escherichia coli* and *Enterobacter*)

· Inoculation loop

· Transfer pipettes

METHOD

A. Inoculation of medium · Prepare MR-VP broth as described above

· Prior to use, allow the medium to come to room temperature.

· Inoculate one tube of MR-VP broth from a fresh (18 to 24 hours) pure culture of the test culture.

· Transfer a light inoculum from an isolated colony and resuspend it in the 5 ml MR-VP broth tube.
· *Escherichia coli* (MR+, VP-) used as control culture.
· Incubate the strains and control cultures both positive and negative at 35°C (+/- 2°C) for 48 hours.

Observation-the mixture is allowed to stand for about 15-30 minutes.

Interpretation-
Positive Result: If the color of the solution becomes pink or red, it means that the organism has the capacity to produce acetoin.
- Result: If solution is still yellow or fails to change color, then no acetoin is formed.
. The VP test is particularly useful for distinguishing between enteric bacteria, such as *Enterobacter aerogenes* (positive) and *Escherichia coli* (-)

CITRATE UTILIZATION TEST

This test is used in the IMViC series also. The goal of this test is to determine whether the strains cam utilize citrate as their sole carbon source. Inorganic ammonium salt is the only source of nitrogen. Where it is used as the source of citrate with which to form carbonates and bicarbonates, the rise in pH was.

MATERIALS REQUIRED

SIMMON CITRATE AGAR COMPOSITION(50 ml)

Constituents(HIMEDIA)	Standard value (g/l)	For experiment (g/ml)
Ammonium dihydrogen phosphate	1	0.09
Magnesium Sulfate	0.2	0.018
Dipotassium phosphate	1	0.09
Sodium citrate	2	0.18
Sodium chloride	5	0.45
Bromothymol blue	0.08	0.0072
Bacteriological agar	15	1.35
Distilled water	1000	90

Adjust pH to 6.9.
· Add agar and bromothymol blue.
· Gently heat, with mixing, to boiling until agar is dissolved.
· Dispense 4.0 to 5.0 ml into 16-mm tubes.

. Autoclave 15 min at 121°C under 15 psi. Chill slantwise (long stare, shallow butt). The sterile medium should be very dark forest green due to the pH of the sample and the bromothymol blue. Inoculation of medium

· Allow tubes to warm up to room temperature prior to inoculation. · Use a fresh (16- to 18-hour) pure culture as an inoculation source.

. Streak a slant lightly with a single isolated colony.

· Avoid using liquid cultures as the inoculum source. · Incubate at 35°C (+/- 2°C) for 18 to 48 hours. Incubation may be extended up to 7 days in some organisms as they grow poorly on citrate medium

Interpretation:

· Citrate positive: there will be dense growth on slant and medium will turn a bright Prussian blue

· Citrate Negative: trace or no growth will be visible.

UREASE TEST

Urease test is used to differentiate between the microorganisms that can hydrolyse urea to produce ammonia and carbon dioxide. This means that the urease enzyme is present in that bacterial strain.

CONSTITUENTS(HIMEDIA)	STANDARD VALUE g/l	FOR EXPERIMENT g/ml
Dextrose	1	0.09
Peptone	1	0.09
Urea	20	1.8
Phenol red	0.012	0.00108
Potassium phosphate monobasic	2	0.09
Sodium chloride	5	0.45
Agar	15	1.35
Distilled water	1000	90

PROCEDURE:

- All the ingredients are mixed properly and autoclaved at 121 psi for 15 mins.
- The slant is prepared and ready for inoculation of the strains. The slants are individually streaked.
- The remaining media components are combined after autoclaving of the agar as mentioned above, due to the fact that urea will decompose if mixed prior to autoclaving.
- The slants are then kept in incubator at 37°C for 16-18 hours. Some strains show slow results and they should be kept for a week for the urease test to complete.
- The colour change of the slant is observed.

- If the medium is pink, means urease is produced which leads to hydrolysis of urea and pH gets increased due to ammonia production. It leads to positive result.
- The result is negative if the colour is not changed at all.

TRIPLE SUGAR IRON TEST(TSI)

This test is used for carbohydrate fermentation and hydrogen sulfide production of strains. It is a differential medium that can distinguish between aerobic and anaerobic strains fermentation of different sugars. The sugars are dextrose ,sucrose and lactose. If any of the carbohydrates are formed then the colour will change as the pH. drops and the colour change from red to yellow.

CONSTITUENTS(HIMEDIA)	STANDARD VALUE g/l	FOR EXPERIMENT g/ml
Beef extract	3	0.36
Peptone	20	2.4
Yeast extract	3	0.36
Dextrose	1	0.12
Lactose	10	1.2
Sucrose	10	1.2
Ferrous sulphate	0.2	0.024
Sodium chloride	5	0.6
Sodium thiosulphate	0.3	0.036
Phenol red	0.024	0.0028
Agar	13	1.56
Distilled water	1000	120

PROCEDURE:

- Use a straight inoculating needle to pick up isolated colony that are fresh streaked the day before.
- Adjust the pH of the media to 7.3 and autoclave.
- Streak slant of TSI. Let excess air to give an open discontinuance.
- Interpretation: If any of the sugar is fermented then the slant changes its colour to yellow indicating the sugar fermentation. Negative result is seen as no change in the medium ,means the colour remains as red due to phenol red indicator.

CATALASE TEST

The catalase test is a biochemical test that is commonly used for identification of organisms that produce the enzyme catalase. This enzyme breaks down hydrogen peroxide (H_2O_2) into water and oxygen, which can be observed as bubbling or foaming when hydrogen peroxide is applied to a culture. This reaction is clearly seen by the bubble formation .

MATERIALS REQUIRED

· 3% hydrogen peroxide
· Grease-free slides
· Dropper
· Inoculation loop

METHOD

Slide (drop) method

· Place a slide and label it with the name of the strain
· Take a drop of saline and make a smear of the strain so that it is completely mixed.
· A dropper is used and one drop of H_2O_2(Hydrogen peroxide) is added to the smear.

Observation: Low light is preferred to see the bubbling formation. If it is a vigorous bubble indicating the organism produces catalase rapidly.

. Weak reactions indicate slow catalase formation.

- Control is taken as- *E.*Coli(- control) and *Staphylococcus aureus* (Positive control).

COAGULASE TEST

This test is used to differentiate a particular strain *Staphylococcus aureus* from other *Staphylococcus* strains. The protein coagulase acts as fibrinogen which causes clumping of the cells of the strains.

MATERIALS REQUIRED

· Saline and Plasma
· Grease-free slides
· Dropper
· Inoculation loop

Procedure-Slide test:

- The pure culture of the strains are used for this test.
- Using a dropper saline is put on the slide and a smear is made/
- A few drops of plasma is put in the slide and checked for clumping.

Observation- If the clot is formed means the presence of coagulase. No clot formation result in - result.

ANTIBIOTIC SUSCEPTIBILITY TEST:

This test is done to determine how sensitive and resistant the bacterial strain is to various antibiotics. It is used so that the treatment is much more useful in the future. The presence and absence of the inhibition zone is used to know the ability to inhibit the organism.

DISK DIFFUSION METHOD(KIRBY-BAUER TEST)

THEORY: The antimicrobial disks after being placed in the Mueller Hilton agar plate will start diffusing in the surroundings. It creates a concentration gradient and the area is formed where the bacteria cannot grow which is called the inhibition zone.

Antibiotic disc used- Ampicillin ,Chloramphenicol ,Methicillin ,Vancomycin and Tetracycline.(HIMEDIA)

PROCEDURE:

- Incubation of the strains is done on MHB(Mueller Hilton Broth) on the night before so that the bacteria is in the log phase when the antibiotic test is done.
- After 16-18 hours the O.D is checked in a microplate reader(Model name-Synergy Biotech Microplate Reader) to see whether the bacteria is in the lop phase or not. The O.D is taken at 600nm. If the absorbance is 0.5 means that the antibiotic test can be done.
- The bacterial strain is spread plated and was kept for 5-10 mins for proper diffusion of the sample in the agar plate.
- The disks were placed and kept in the incubator for 16-18 hours at 37°C.
- Inhibition zones are measured after incubation.
  Interpretation: The diameter of the inhibition zones is measured to classify bacteria as susceptible, intermediate, or resistant based on standardized guidelines.

SIDEROPHORE ASSAY

Siderophore are iron chelating potential metabolites. They have high affinity ligand that can bind ferric ions and transport through the cell membrane. Although Iron is a micronutrient, its availability is essential as it is important in various biological processes. Iron forms insoluble complexes that is the reason of iron deficiency. Some microbial strains strongly chelate iron and form iron-siderophore complexes. The ability to form siderophore is commonly detected by CAS(Chrome Azurol Sulphonate). In CAS assay there is a competition between the iron uptake in the siderophore and the ferric complex present in the CAS dye. Siderophore strongly chelate the iron from the CAS iron-dye complex and the dye then changes the colour in the media as it becomes free from bluish purple to orange. The measurement of siderophore production is taken by spectrophotometric analysis. The 96 well plate is used and the O.D is taken for the analysis.

Procedure:

PREPERATION OF MM9 MEDIA

CONSTITUENTS(Company name-MERCK)	COMPOSITION(in g/ml)
Dextrose	5
Potassium dihydrogen phosphate	0.5
Magnesium Chloride	0.1
Ammonium Sulphate	0.5
Calcium Chloride	5.549
PIPES	1.66
Sodium Chloride	2.5
Dipotassium Hydrogen Phosphate	0.008
Distilled water	500 ml

- The strains are inoculated 1.5 ml Eppendorf(TARSON) in MM9 media(used because it a minimal media that lack iron).
- They are kept in the incubator for 48 hours at 37°C.
- The Eppendorf(TARSON) are centrifuged(Model name- spin Win) at 10,000 rpm for 7 mins and the supernatant is taken.
- They are triplicated in 96 well plate(100ul each) and 100ul CAS media is added to the sample.
- A shuttle(Sulpho salicylic acid) is added(10 ul) to transfer the iron from the CAS dye complex to siderophore as not all siderophores are capable for the iron chelating from the complex of CAS-Iron complex.
- The whole experiment should be done in the dark. Wrap the 96 well plate in aluminium foil and keep in the incubator for 1 hour at 37°C.
- The O.D is taken at 630nm using microplate reader(Model name-Synergy Biotech Microplate Reader) and the analysis is done

Siderophore production (psu)≡(Ar -As)* 100/A_r

Where A_r=absorbance of the uninoculated broth and CAS solution

A_s= the sample solution with the CAS media

Observation: All the strains showed positive result meaning they showed orange coloured product which means that siderophore production occurred in the bacterial strains.

DPPH Radical Scavenging Assay(Company name- Thermoscientific)

This assay is done to check the antioxidant activity in the bacteria strains for different groups. Antioxidants can prevent or delay the oxidation of substrate molecules. They donate electrons free radicals to neutralize and minimize oxidative damage. Free radicals create a compelling damage to the human body. When a solution has the capability to donate a hydrogen ion the intense purple colour of DPPH is reduced to pale yellow colour. The free radical gets reduced from the antioxidants and can be measured by UV-VIS Spectroscopy.(79)

Procedure:

i. The bacterial strains are inoculated in BHI(Brain Heart Infusion) broth and kept in the incubator at 37°C for 16-18 hours.
ii. The strains are centrifuged(Model name-SpinWin) for 10,000rpm for 7 mins and the bacterial supernatant is taken
iii. 50ul of each strain is taken and triplicated it in the 96 well plate.
iv. Then DPPH is added in a dark room environment because it is light sensitive. Then the plate is wrapped in foil and kept it in the incubator at 28°C for 30 mins,
v. Then the O.D is taken at 517nm with the microplate reader(Model name-Synergy Biotech Microplate Reader) and graph is prepared for see the percentage of radical scavenging assay production

RSA(%) = (Ac – As/ Ac) × 100

Ac is the absorbance at 517 nm of the control sample, and As is the absorbance at 517 nm that contains the bacterial strains sample.

Observation: All the strains changed the colour to yellow means that the radical got scavenged and antioxidant activity is seen in the strains

MacConkey agar differentiation

It is a differential and selective media which helps us to differentiate between gram negative bacteria that are lactose fermenters with non-lactose fermenters.

Procedure:

a) Gram negative rods are taken and streaked on the plate with *E.coli* as a control.

Observation-One strain ferments lactose and changed the colour from neutral red to light red colouration.

BIOFILM ASSAY

This assay is done to see if the microbes have the ability to form biofilm to survive in harsh gut conditions

Procedure:

a) TSB Media(Himedia) is prepared(100 ml) and the bacterial strains are inoculated for 16-18 hours.

b) Then in a 96 well plate. Add 100ul of the strains and triplicate it .Then add control like *E.Coli* and *S.Aureus*.

c) Keep it in the incubation for 72 hours

Staining:

i. After the incubation,the cells are dumped out from the well plate.
ii. Then gently submerge the plate with water and do it for 2 times to remove the unattached cells completely and leftover media components .
iii. Add 125ul of 0.1% solution of Crystal Violet in each well plate.
iv. Incubate the plate at room temperature for 10-15 minutes.
v. Rinse the plate for at least 4-5 times to get rid of the excess cells and the dye. The well plate is turned upside down and let it dry for overnight
vi. To solubilize the CV, add 125ul of 30% acetic acid in water to each well of the microtiter plate
vii. Incubate the microtiter plate at room temperature for 10-15 mins.
viii. 125ul of the solubilized CV was transferred to new microtiter dish
ix. Absorbance is measured at 550nm using 30% Acetic acid in water as blank.

Analysis of Haematological parameters

The haemoglobin was measured on days 1,3 and 5 after phenyl hydrazine was injected.On the day of sacrifice which is day 7 ,also the RBC count was done and blood was taken to see the percentage of haemoglobin and haematocrit was measured.

Procedure:

i. The mice was anaesthetized and the tail vein was pricked to collect the blood for the RBC cell count and to see the haematocrit level.
ii. For RBC counting, the blood sample was diluted and placed on the haemocytometer glass slide.
iii. Then it was covered with a cover slip to see under a microscope.
iv. The cells are counted in specialized grids .

HISTOLOGY OF THE GUT

Histology is the study where the basic structure is studied. They are most commonly seen and analysed under a microscope after staining procedures. There are many steps for preparation of the histological samples for analysis.

- After the mice is euthanized, the ileum and colon of the 4 groups(control, anaemia, anaemia+nanoparticle and only nanoparticle) was kept in Bouin's solution to preserve the structure as it is.Bouin's solution is used for soft and delicate tissues like the gut(ileum and colon) so as to preserve the tissue and prevent autolysis. The mucosal linings and intestinal villi are delicate so any harsh fixatives cannot be used.

TISSUE FIXATION

a. The tissue is placed in Bouin's solution for duration 6-24 hours.
b. After 30 mins dip the tissue in fresh fixative (1:10 volume)
c. Keep if for rest of the fixation period

DEHYDRATION

a. Transfer the tissue to 50% alcohol and keep if for 45 mins
b. Transfer the tissue to 70% alcohol and keep if for 45 mins
c. Transfer the tissue to 80% alcohol and keep if for 45 mins
d. Transfer the tissue to 90% alcohol and keep if for 45 mins
e. Transfer the tissue to 100% alcohol and keep if for 30 mins. Proceed for 2 times

CLEARING

a. Next transfer the tissue into benzene for 20 mins and we have to change 3 times.
b. Then transfer the tissues in benzene/paraffin for 30 mins and then overnight incubation
c. Prepare a boat for the block preparation.

BLOCK PREPARATION

a. Melted paraffin is poured on the boat prepared.
b. Slowly the tissues are transferred and properly aligned for the sectioning later.
c. Place the boat in water and keep it for 30 mins for the solidification of the block. Store the block at 4°C.

SECTIONING

The tissue block is not cut and placed on a holder in a machine called a microtome. They are cut at extremely thin sections like 4-micron size. They are now placed in a water bath with temperature 52-54°C. The tissue gets expanded and the tissue is taken in a slide that is smeared with Meyers albumin solution.

STAINING(H and E staining)

H&E is the combination of two most commonly used histological stains: haematoxylin and eosin. The haematoxylin stains nuclei a purple bluish colour, and eosin stains the cytoplasm pink, while other takes different hues of the stain.

PROCEDURE

a. Deparaffinization- The sectioned tissue slide is put in xylene for 3 mins to get rid of the paraffin.

b. Hydration- Next the tissue is placed in 100% ethanol for 30 seconds, then in 70% ethanol for 30 sections and placed in distilled water for 1.5 minutes.

c. Staining- The slide is next stained with haematoxylin for 1.5 minutes. Then the slide is washed in distilled water for 3-4 times to get rid of the extra stain.

d. Bluening-The slide is then kept in tap water for 15 minutes so that the stains gets neutralized a darker colour gets appeared.

e. Dehydration- Now the tissue is placed in 70% ethanol for 30 sec(2 times). The same is done in 90% ethanol and 100% ethanol.

f. Second staining- Eosin is added and the slide is kept in rocking motion for 30s for proper staining. Then the slide is washed with absolute alcohol wash to get rid of the extra stain.

g. Clearing- The slide is then placed in xylene to get rid of the extra debris left and is done till the murkiness gets removed.

h. Then the slide is dried and a mounting solution is placed(DPX).A cover slip is put and now the slide is ready to be viewed under a microscope.

4. RESULTS AND DISCUSSION

1. SPREAD PLATE PICTURES

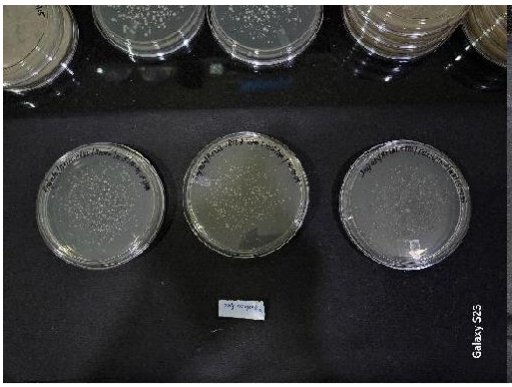


FIG-4.1.1- Only nanoparticle

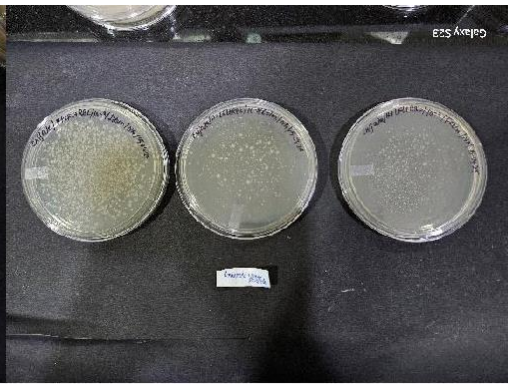


FIG-4.1.2- Anaemia+nanoparticle

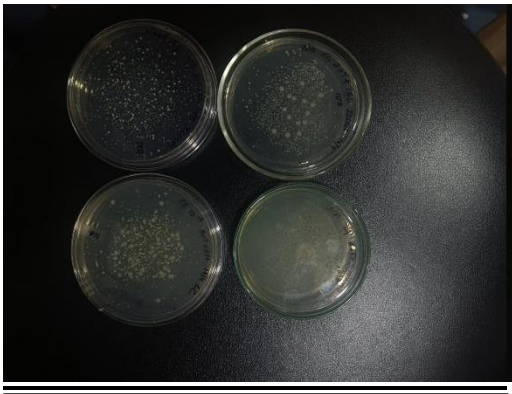


FIG-4.1.3- Only anaemia

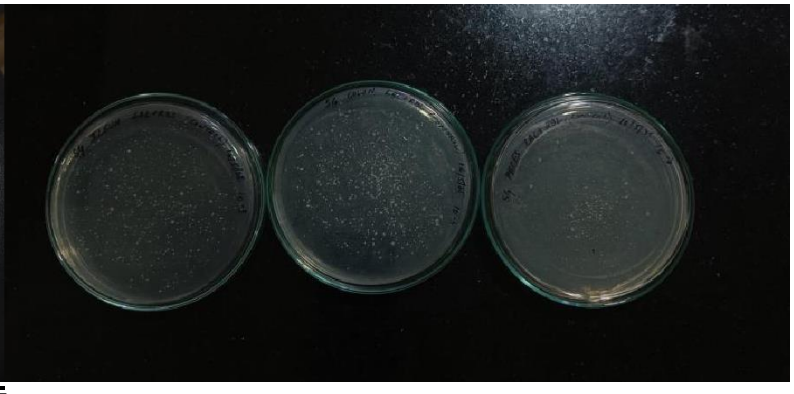


FIG-4.1.3- Control

COLONY MORPHOLOGY[Table -4.1]

TYPE	STRAIN NAME	SHAPE and COLOUR	ELEVATION	MARGIN
B+T+RFL(F) -S	Anaemic mice	white, round	Convex	Entire
B+T+RFL(F) -W	Anaemic mice	White, irregular	Flat	Entire
B+T+RFL(F) -C	Anaemic mice	Round, coloured	Flat	Entire
B+T+RFL(F) -LW	Anaemic mice	Round, white	Flat	Entire
H+LFL+RBL(IL)-TW	Anemic+nanoparticle mice	Round, Transparent white	Flat	Entire
H+LFL+RBL(IL)-BW	Anemic+nanoparticle mice	Round, white	flat	Entire
H+LFL+RBL(IL)-C/CW	Anemic+nanoparticle mice	Round, coloured	Flat	Entire
H+LFL+RBL(IL)-S	Anemic+nanoparticle mice	Round, white	Flat	Entire
H+LFL+RBL(CO)-BW	Anemic+nanoparticle mice	Round, white	Flat	Entire
H+LFL+RBL(CO)-SW	Anemic+nanoparticle mice	Round, small white	Flat	Entire
B+T+LBL(F)-S.1.1	anaemic mice	Round, small	Flat	Entire
B+T+LBL(F)-W.1	Anaemic mice	Round, small	Flat	Entire
H+LBL+RBL(CO)-W.2	only nanoparticle mice	round	Flat	Entire
H+LBL+RBL(CO)-SW.1	only nanoparticle mice	Small,white,round	Flat	Entire
B+LBL+RBL(IL)-SW.1	only nanoparticle mice	Small,white,round	Flat	Entire
B+LBL+RBL(IL)-C.2	only nanoparticle mice	Small,coloured,round	Flat	Entire
H+LFL+RBL(F)-SW.2	Anemic+nanoparticle mice	Small, round	Flat	Entire
H+LFL+RBL(F)-TW.2	Anemic+nanoparticle mice	Small, round	Flat	Entire

H+LBL+RBL(F)-TW.2	only nanoparticle mice	Small, round, transparent white	Flat	Entire
H+LBL+RBL(F)-C.3	only nanoparticle mice	Round, coloured	Flat	Undulate
LBL+RBL(IL)	Anaemic mice	White, round	convex	Entire
B+T+LBL(IL)-IWB	Anaemic mice	White, round	flat	Undulate
B+T+LBL(IL)-IY	Anaemic mice	Yellow, round	flat	undulate
B+T+RFL(IL)-IO	Anaemic mice	Orange, round	flat	Entire
B+T+RFL(IL)-IY	Anaemic mice	Yellow, round	flat	Entire
B+T+RFL(CO)-CO	Anaemic mice	Orange, round	flat	Entire
B+T+RFL(CO)-CY	Anaemic mice	Yellow, round	flat	Entire
B+T+LBL(CO)-CY	Anaemic mice	Yellow, round	flat	Entire
LBL+RBL(IL)-IWB	Control mice	White ,round	convex	Entire
LBL+RBL(IL)-IWS	Control mice	White ,round	flat	Entire
LBL+RBL(CO)-CWB	Control mice	White ,round	flat	Entire
LBL+RBL(CO)-CWS	Control mice	White ,round	flat	Entire
LBL+RBL(F)-FWB	Control mice	White ,round	flat	Entire
LBL+RBL(F)-FWS	Control mice	White ,round	flat	Entire

2. COLONY ISOLATED PLATE PICTURES

FIG-4.2.1- The pure colony isolation plates

3. GRAM STAINING RESULTS AND PICTURES(Table 4.2)

STRAIN NAME	GRAM STAINING	
B+T+RFL(F) -S	GRAM POSITIVE COCCUS	Anaemic mice
B+T+RFL(F) -W	GRAM POSITIVE COCCUS	Anaemic mice
B+T+RFL(F) -C	GRAM POSITIVE COCCUS	Anaemic mice
B+T+RFL(F) -LW	GRAM NEGTIVE BACILLUS	Anaemic mice
H+LFL+RBL(IL)-TW	GRAM POSITIVE COCCUS	Anemic+nanoparticle mice
H+LFL+RBL(IL)-BW	GRAM - RODS	Anemic+nanoparticle mice
H+LFL+RBL(IL)-C/CW	GRAM - COCCUS	Anemic+nanoparticle mice
H+LFL+RBL(IL)-S	GRAM - COCCUS	Anemic+nanoparticle mice
H+LFL+RBL(CO)-BW	GRAM - COCCUS	Anemic+nanoparticle mice
H+LFL+RBL(CO)-SW	GRAM POSITIVE COCCUS	Anemic+nanoparticle mice
B+T+LBL(F)-S.1.1	GRAM - RODS	anaemic mice
B+T+LBL(F)-W.1	GRAM POSITIVE COCCUS	Anaemic mice
H+LBL+RBL(CO)-W.2	GRAM POSITIVE COCCUS	only nanoparticle mice
H+LBL+RBL(CO)-SW.1	GRAM POSITIVE COCCUS	only nanoparticle mice
B+LBL+RBL(IL)-SW.1	GRAM POSITIVE COCCUS	only nanoparticle mice
B+LBL+RBL(IL)-C.2	GRAM POSITIVE COCCUS	only nanoparticle mice
H+LFL+RBL(F)-SW.2	GRAM - SMALL RODS/COCCUS	Anemic+nanoparticle mice
H+LFL+RBL(F)-TW.2	GRAM POSITIVE COCCUS	Anemic+nanoparticle mice
H+LBL+RBL(F)-TW.2	GRAM POSITIVE COCCUS	only nanoparticle mice
H+LBL+RBL(F)-C.3	GRAM POSITIVE COCCUS	only nanoparticle mice
LBL+RBL(IL)	POSITIVE COCCUS	Anaemic mice
B+T+LBL(IL)-IWB	NEGATIVE RODS	Anaemic mice
B+T+LBL(IL)-IY	POSITIVE COCCUS	Anaemic mice
B+T+RFL(IL)-IO	NEGATIVE COCCUS	Anaemic mice
B+T+RFL(IL)-IY	POSITIVE RODS	Anaemic mice
B+T+RFL(CO)-CO	POSITIVE COCCUS	Anaemic mice
B+T+RFL(CO)-CY	POSITIVE COCCUS	Anaemic mice
B+T+LBL(CO)-CY	NEGATIVE COCCUS	Anaemic mice
LBL+RBL(IL)-IWB	POSITIVE COCCUS	Control mice
LBL+RBL(IL)-IWS	POSITIVE COCCUS	Control mice
LBL+RBL(CO)-CWB	NEGATIVE COCCUS	Control mice

	LBL+RBL(CO)-CWS	POSITIVE COCCUS	Control mice	
	LBL+RBL(F)-FWB	NEGATIVE COCCUS	Control mice	
	LBL+RBL(F)-FWS	POSITIVE COCCUS	Control mice	

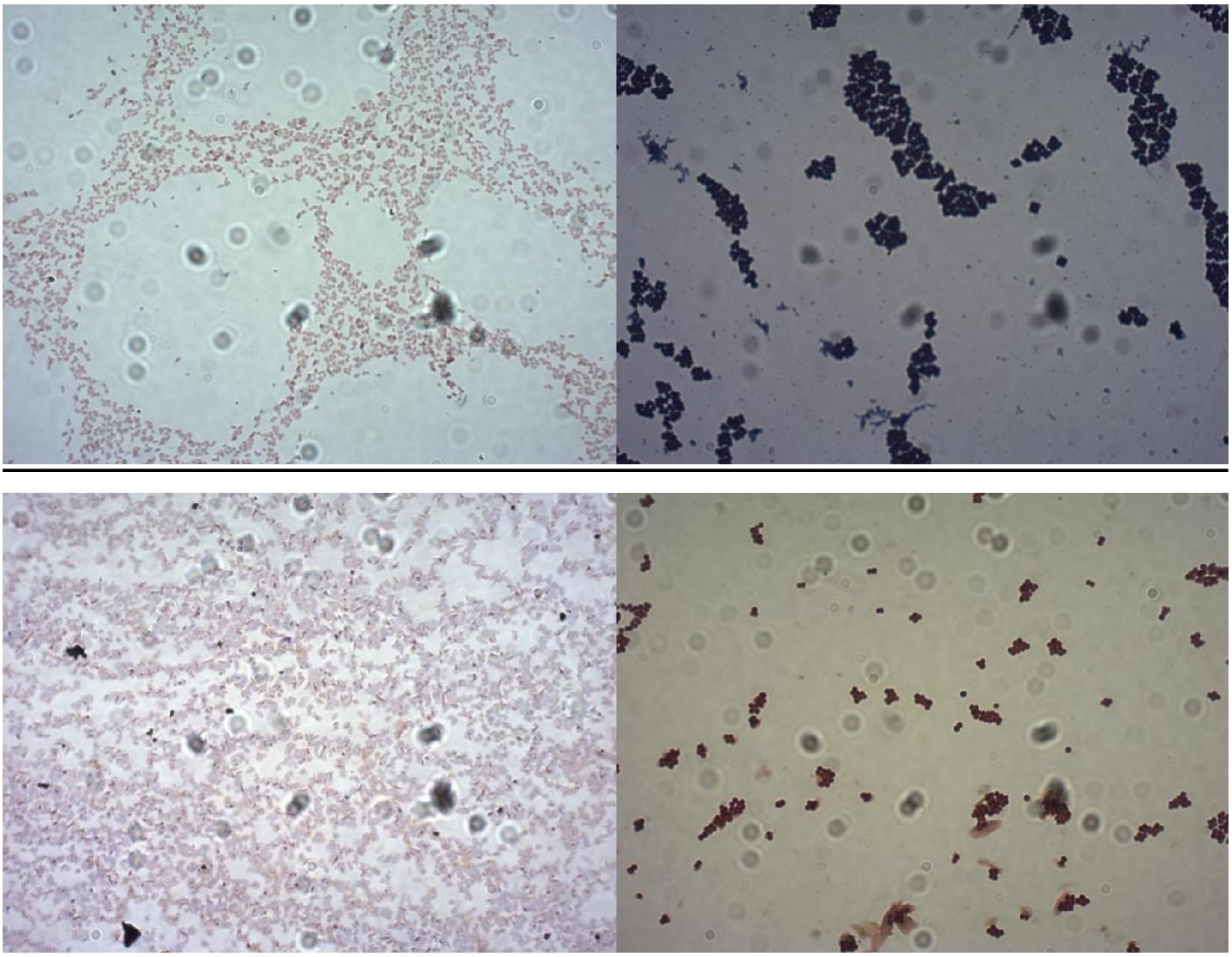


FIG-4.3.1-From the top left-Gram - bacillus, gram positive coccus, gram - rods and gram negative coccus is found in anaemic mice

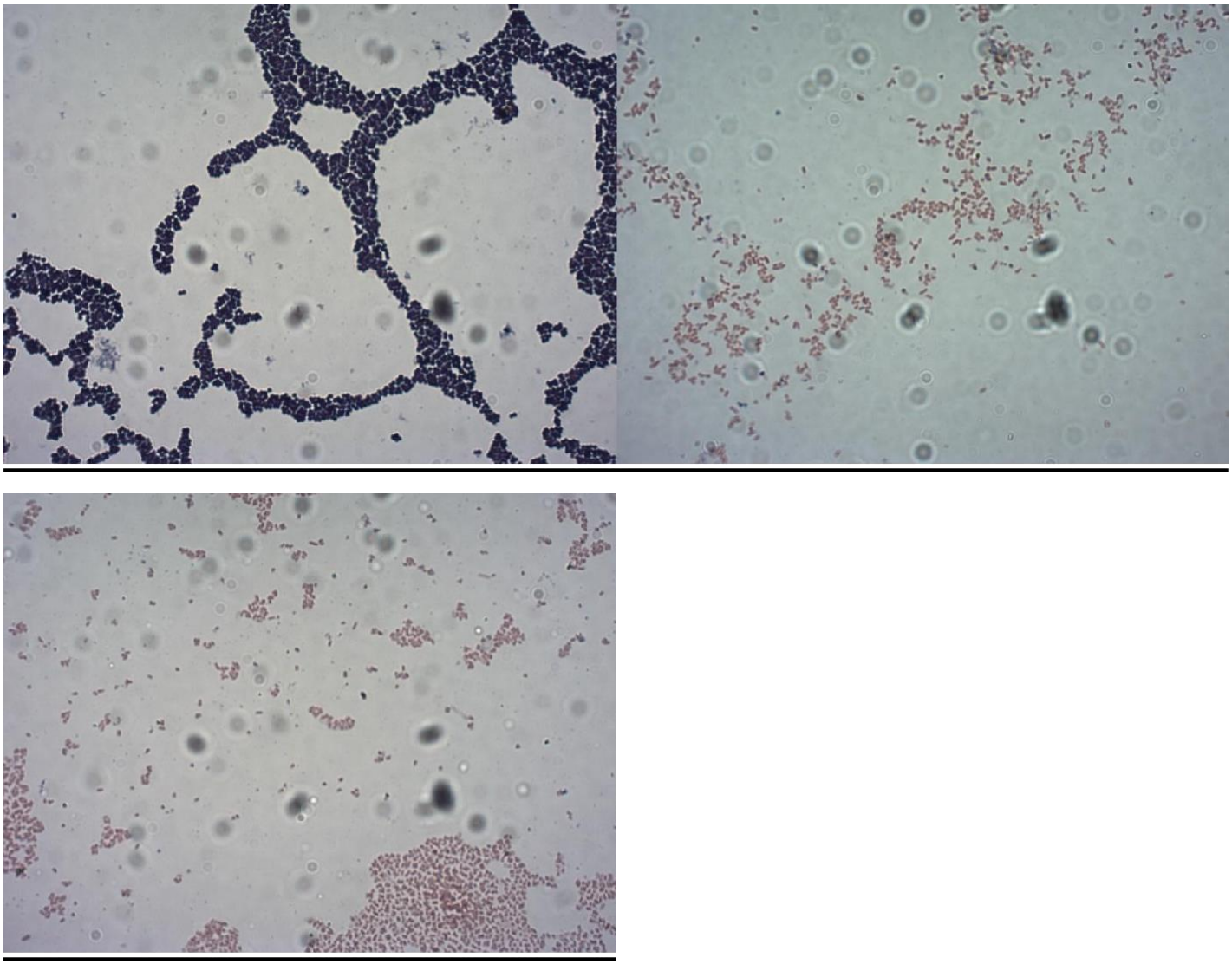


FIG 4.3.2-From the top left-Gram positive coccus, gram - rods and gram - coccus is found in anaemic+nanoparticle mice

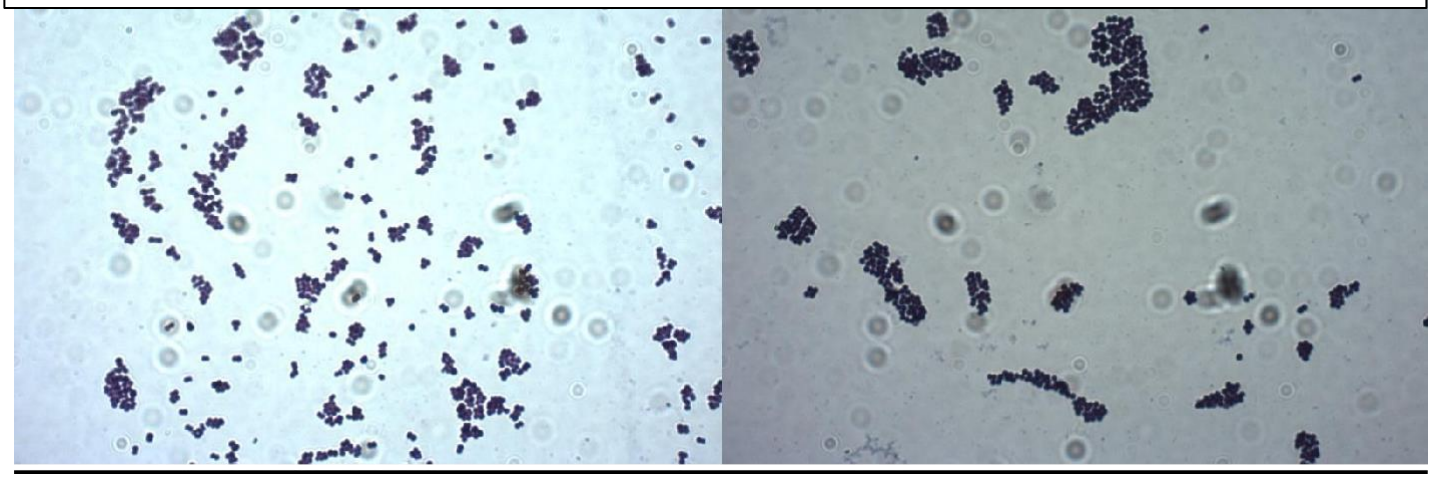


FIG-4.3.3-Only gram-positive coccus is found in only nanoparticle mice

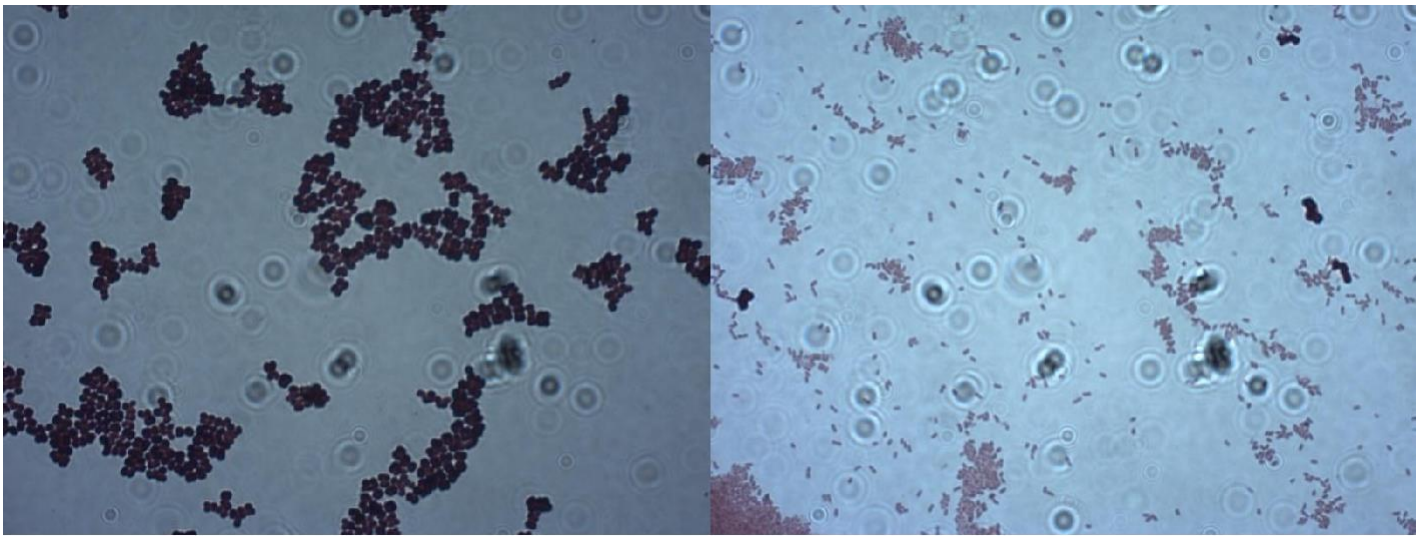


FIG-4.3.4-Only gram positive and gram - coccus is found in only nanoparticle mice

4. IMViC PICTURES

A. INDOLE TEST

FIG-4.4.A.1- The indole result for some of the strains with the control.

B. . METHYL RED TEST

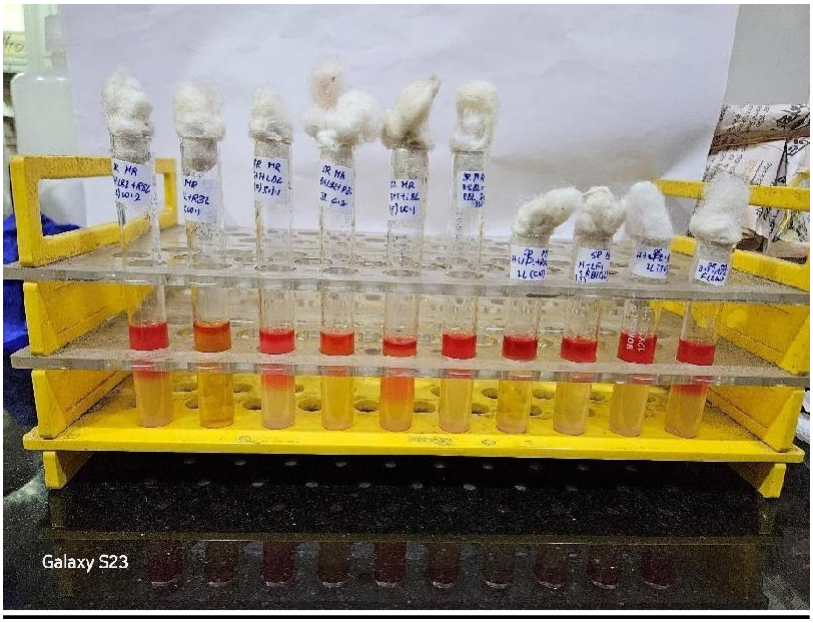


FIG - 4.4.B.1- The methyl red results with control

C. VOGUES PROSKAEUR TEST

FIG- 4.4.C.1- The Vogue Proskauer results with control

STRAIN NAME	**IMViC results**

		Indole test	MR test	VP test
B+T+RFL(F) -S	Anaemic mice	-	+	-
B+T+RFL(F) -W	Anaemic mice	-	+	-
B+T+RFL(F) -C	Anaemic mice	-	+	-
B+T+RFL(F) -LW	Anaemic mice	+	+	-
H+LFL+RBL(IL)-TW	Anemic+nanoparticle mice	+	+	-
H+LFL+RBL(IL)-BW	Anemic+nanoparticle mice	+	+	-
H+LFL+RBL(IL)-C/CW	Anemic+nanoparticle mice	+	+	-
H+LFL+RBL(IL)-S	Anemic+nanoparticle mice	+	+	-
H+LFL+RBL(CO)-BW	Anemic+nanoparticle mice	+	+	-
H+LFL+RBL(CO)-SW	Anemic+nanoparticle mice	+	+	-
B+T+LBL(F)-S.1.1	anaemic mice	+	+	-
B+T+LBL(F)-W.1	Anaemic mice	+	+	-
H+LBL+RBL(CO)-W.2	only nanoparticle mice	+	+	-
H+LBL+RBL(CO)-SW.1	only nanoparticle mice	-	+	+
B+LBL+RBL(IL)-SW.1	only nanoparticle mice	+	+	+
B+LBL+RBL(IL)-C.2	only nanoparticle mice	-	+	-
H+LFL+RBL(F)-SW.2	Anemic+nanoparticle mice	-	+	+
H+LFL+RBL(F)-TW.2	Anemic+nanoparticle mice	-	+	+
H+LBL+RBL(F)-TW.2	only nanoparticle mice	-	+	+
H+LBL+RBL(F)-C.3	only nanoparticle mice	-	+	+
LBL+RBL(IL)	Anaemic mice	-	+	-
B+T+LBL(IL)-IWB	Anaemic mice	-	-	+
B+T+LBL(IL)-IY	Anaemic mice	-	+	+
B+T+RFL(IL)-IO	Anaemic mice	-	+	+
B+T+RFL(IL)-IY	Anaemic mice	-	-	+
B+T+RFL(CO)-CO	Anaemic mice	-	+	+
B+T+RFL(CO)-CY	Anaemic mice	-	-	+
B+T+LBL(CO)-CY	Anaemic mice	-	-	-
LBL+RBL(IL)-IWB	Control mice	-	+	-
LBL+RBL(IL)-IWS	Control mice	-	-	-
LBL+RBL(CO)-CWB	Control mice	-	-	+
LBL+RBL(CO)-CWS	Control mice	-	-	+
LBL+RBL(F)-FWB	Control mice	-	-	-
LBL+RBL(F)-FWS	Control mice	-	+	-

D. CITRATE UTILIZATION TEST

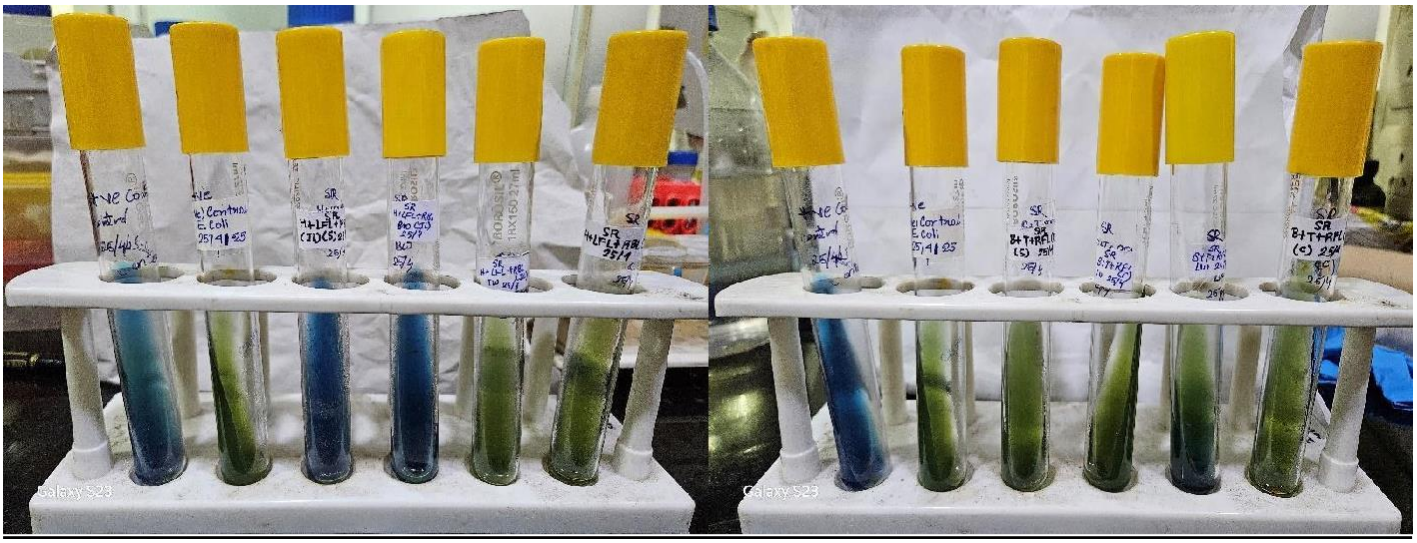
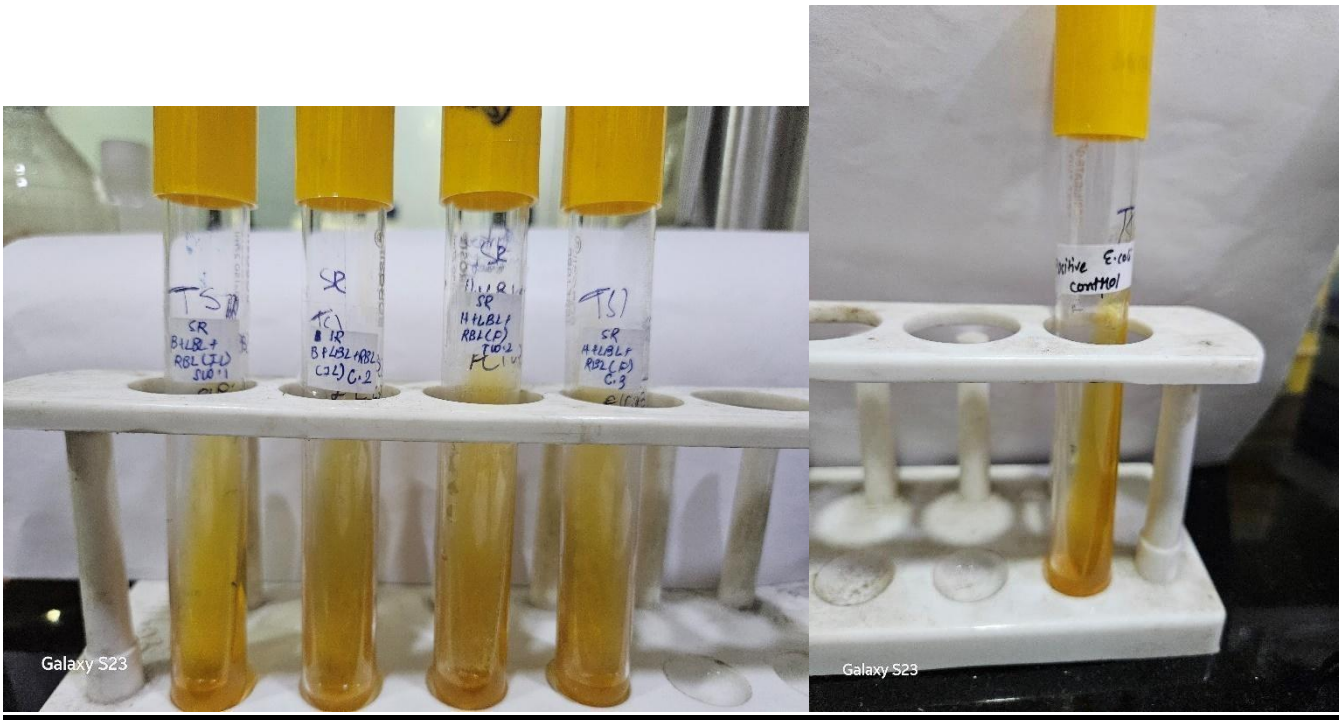


FIG- 4.4.D.1-The citrate results with control.

E. TSI (TRIPLE SUGAR IRON) TEST



F. UREASE TEST

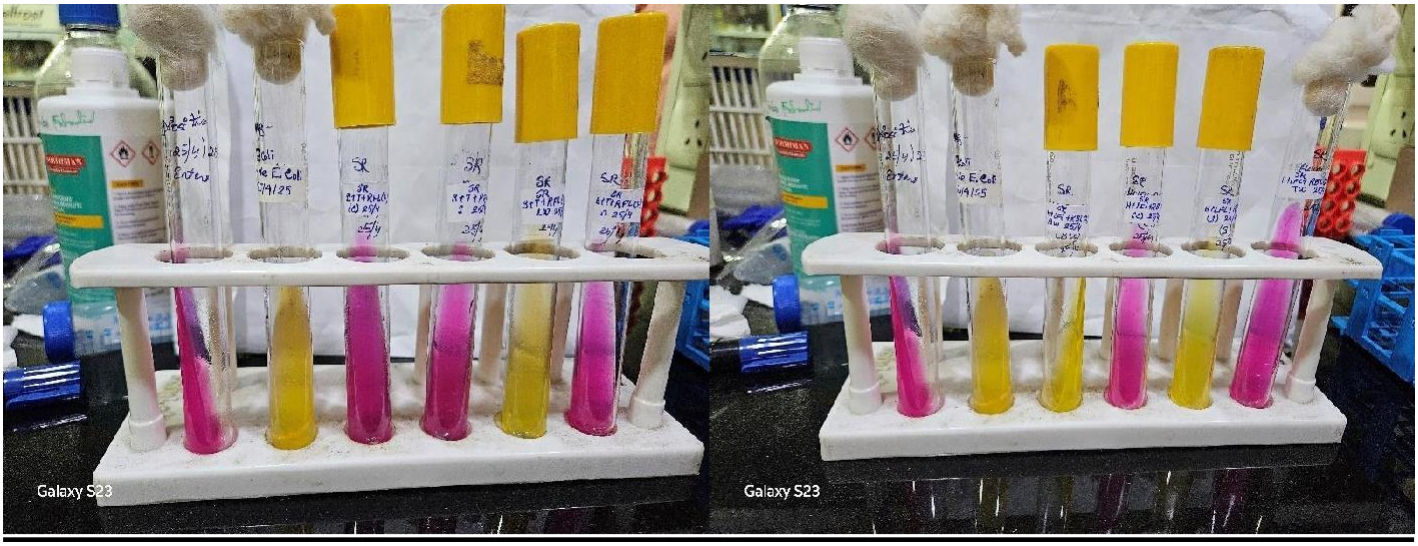


FIG 4.4.F.1- The Urease result with control

STRAIN NAME	IMViC results

		Citrate test	Urease test	TSI test	Catalase	Coagulase
B+T+RFL(F) -S	Anaemic mice	-	+	+	-	+
B+T+RFL(F) -W	Anaemic mice	-	++(1hr)	+	++	+
B+T+RFL(F) -C	Anaemic mice	-	+	+	++	+
B+T+RFL(F) -LW	Anaemic mice	-	- Day 5 and more-+	+	-	–
H+LFL+RBL(IL)-TW	Anemic+nanoparticle mice	-	+	+	-	–
H+LFL+RBL(IL)-BW	Anemic+nanoparticle mice	+	-	+	-	–
H+LFL+RBL(IL)-C/CW	Anemic+nanoparticle mice	-	+	+	++	–
H+LFL+RBL(IL)-S	Anemic+nanoparticle mice	+	+	+	++	–
H+LFL+RBL(CO)-BW	Anemic+nanoparticle mice	+	-	+	+	+
H+LFL+RBL(CO)-SW	Anemic+nanoparticle mice	-	Day 3 - showing + changes >5days-+	+	-	–
B+T+LBL(F)-S.1.1	anaemic mice	-	-	+	+	+

B+T+LBL(F)-W.1	Anaemic mice	-	-	+	+	++
H+LBL+RBL(CO)-W.2	only nanoparticle mice	-	+	+	-	_
H+LBL+RBL(CO)-SW.1	only nanoparticle mice	-	+	+	+	_
B+LBL+RBL(IL)-SW.1	only nanoparticle mice	-	-	+	-	_
B+LBL+RBL(IL)-C.2	only nanoparticle mice	-	+	+	-	+
H+LFL+RBL(F)-SW.2	Anemic+nanoparticle mice	-	-	+	+	_
H+LFL+RBL(F)-TW.2	Anemic+nanoparticle mice	-	+	+	+	+
H+LBL+RBL(F)-TW.2	only nanoparticle mice	-	slightly +	+	+	_
H+LBL+RBL(F)-C.3	only nanoparticle mice	-	slightly +	+	+	_
LBL+RBL(IL)	Anaemic mice	-	-	-	-	-
B+T+LBL(IL)-IWB	Anaemic mice	-	-	+	+	+
B+T+LBL(IL)-IY	Anaemic mice	-	+	+	+	_
B+T+RFL(IL)-IO	Anaemic mice	-	+	+	-	+
B+T+RFL(IL)-IY	Anaemic mice	-	-	-	+	+
B+T+RFL(CO)-CO	Anaemic mice	-	+	+	-	+
B+T+RFL(CO)-CY	Anaemic mice	-	-	-	+	+
B+T+LBL(CO)-CY	Anaemic mice	-	+	+	-	+
LBL+RBL(IL)-IWB	Control mice	-	+	-	-	+
LBL+RBL(IL)-IWS	Control mice	-	+	+	++	+
LBL+RBL(CO)-CWB	Control mice	-	+	-	++	+
LBL+RBL(CO)-CWS	Control mice	-	+	-	++	_
LBL+RBL(F)-FWB	Control mice	-	-	+	-	_
LBL+RBL(F)-FWS	Control mice	-	+	+	++	_

TABLE 4.4.2-The citrate ,urease and tsi test respectively

G. CATALASE TEST

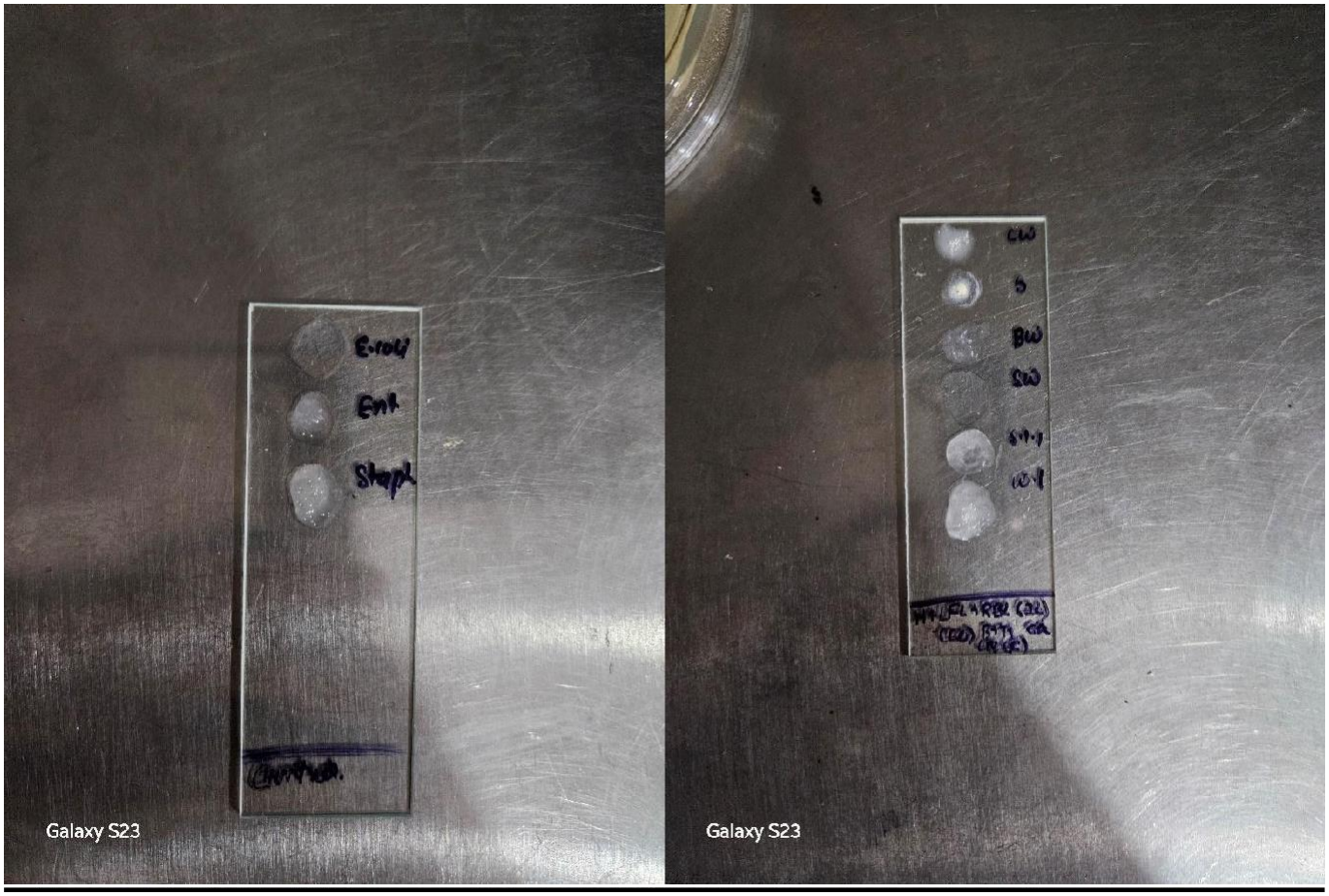


FIG 4.4.G.1- catalase strains with control

H. COAGULASE TEST

FIG 4.4.H.1- Result of coagulase with control

5.4 ANTIBIOTIC SUSCEPTIBILITY TEST(KIRBY-BAUER TEST)

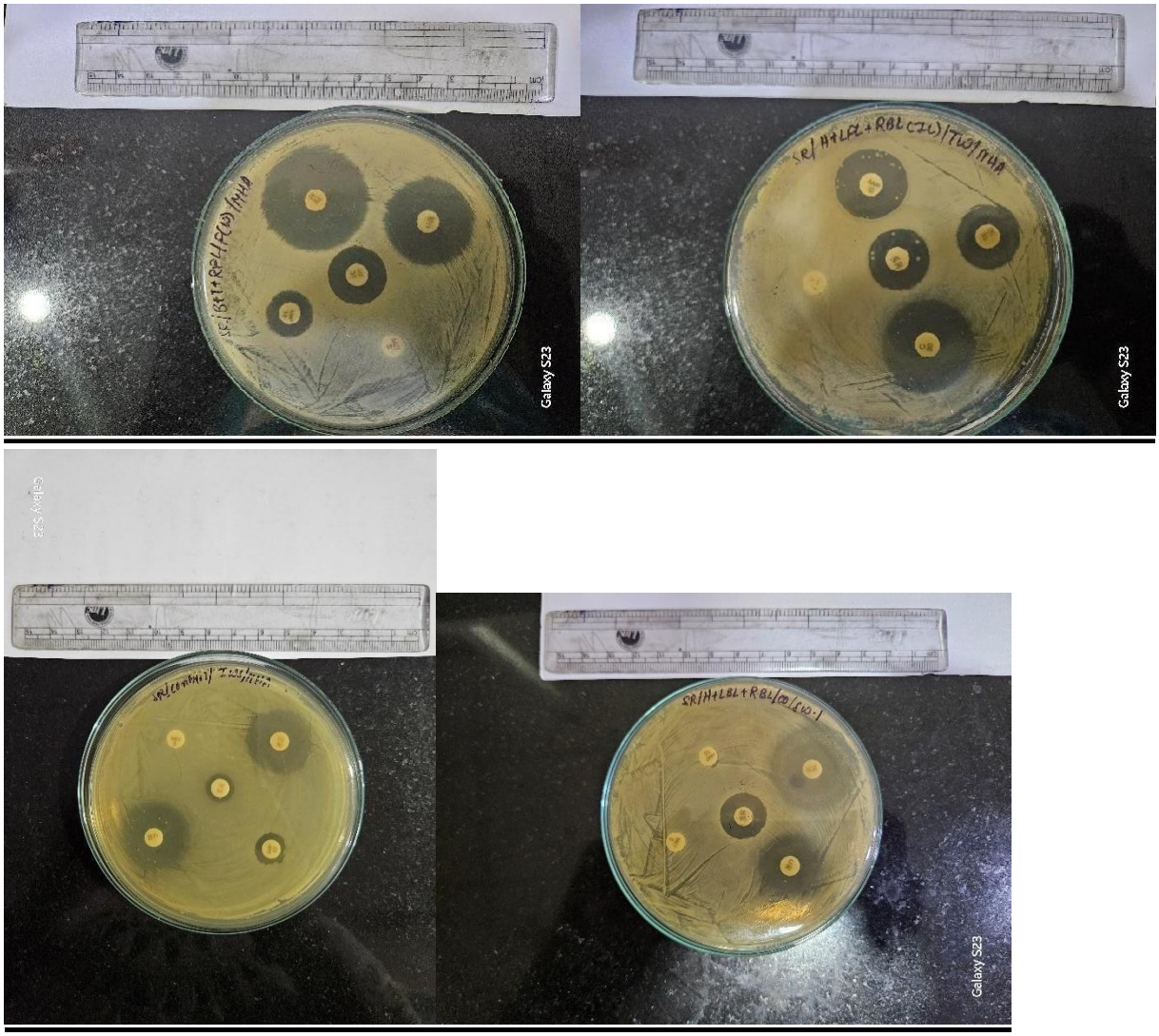


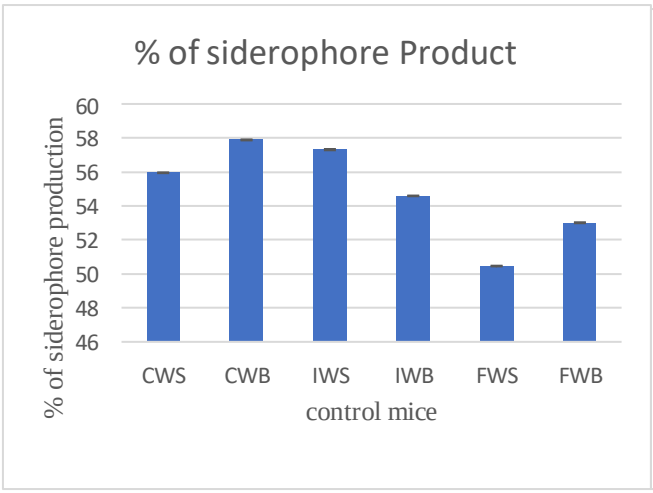
FIG 4.4.1- Antibiotic susceptibility test for top left- anaemic,anaemia+nanoparticle,only nanoparticle and control mice

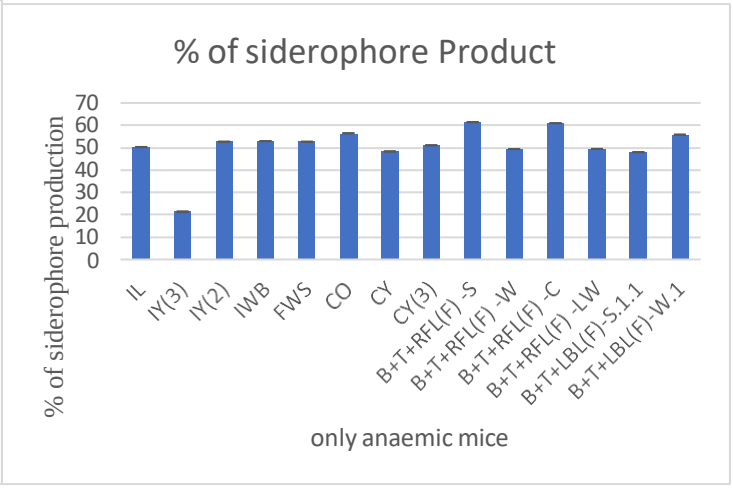
STRAIN NAME		Zone of Inhibition(In cm)				
		TETRACYCLIN	CHLORAMPHENICOL	VANCOMYCIN	METHICILIN	AMPHICILIN
B+T+RFL(F) -S	Anaemic mice	1.597	0.952	1.207	0	0
B+T+RFL(F) -W	Anaemic mice	2.837	2.510	1.661	0	1.231
B+T+RFL(F) -C	Anaemic mice	2.946	2.830	1.650	0	1.654
B+T+RFL(F) -LW	Anaemic mice	2.003	2.663	0.786	0	0
H+LFL+RBL(IL)-TW	Anemic+nanoparticle mice	0.815	1.275	0.637	0	1.188
H+LFL+RBL(IL)-BW	Anemic+nanoparticle mice	2.405	2.317	0	0	0
H+LFL+RBL(IL)-C/CW	Anemic+nanoparticle mice	0	2.795	1.592	0	0
H+LFL+RBL(IL)-S	Anemic+nanoparticle mice	0	2.316	0	0	0
H+LFL+RBL(CO)-BW	Anemic+nanoparticle mice	2.115	2.105	0.921	0	0
H+LFL+RBL(CO)-SW	Anemic+nanoparticle mice	2.015	2.041	0.871	0	0
B+T+LBL(F)-S.1.1	anaemic mice	3.320	1.397	1.883	0	1.097
B+T+LBL(F)-W.1	Anaemic mice	1.925	1.901	0.923	0	0
H+LBL+RBL(CO)-W.2	only nanoparticle mice	2.318	2.136	0	0	0
H+LBL+RBL(CO)-SW.1	only nanoparticle mice	2.581	1.839	1.266	0	0
B+LBL+RBL(IL)-SW.1	only nanoparticle mice	1.706	1.790	0.643	0	0
B+LBL+RBL(IL)-C.2	only nanoparticle mice	2.772	1.986	1.395	0.728	0.797
H+LFL+RBL(F)-SW.2	Anemic+nanoparticle mice	1.621	1.482	0.616	0	0
H+LFL+RBL(F)-TW.2	Anemic+nanoparticle mice	2.292	1.858	1.168	0	0.637
H+LBL+RBL(F)-TW.2	only nanoparticle mice	1.847	2.046	0.943	0	0.732
H+LBL+RBL(F)-C.3	only nanoparticle mice	2	1.817	0.884	0	0
LBL+RBL(IL)	Anaemic mice	0	0	0	0	0
B+T+LBL(IL)-IWB	Anaemic mice	1.803	2.272	1.233	0	1.174
B+T+LBL(IL)-IY	Anaemic mice	2.787	2.529	1.668	0	0.988

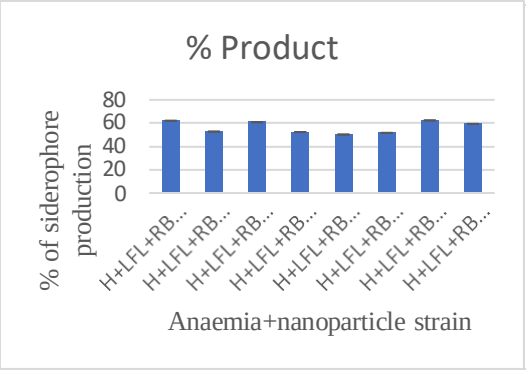
B+T+RFL(IL)-IO	Anaemic mice	2.774	1.917	1.751	0	1.372
B+T+RFL(IL)-IY	Anaemic mice	2.091	1.693	1.398	0	0.776
B+T+RFL(CO)-CO	Anaemic mice	2.289	2.161	1.345	0	1.041
B+T+RFL(CO)-CY	Anaemic mice	2.178	2.098	1.234	0	1
B+T+LBL(CO)-CY	Anaemic mice	2.072	1.693	1.258	0	1.028
LBL+RBL(IL)-IWB	Control mice	1.108	1.846	0.763	0	0
LBL+RBL(IL)-IWS	Control mice	2.016	2.204	0.609	0	0.756
LBL+RBL(CO)-CWB	Control mice	2.622	2.392	1.592	0	0.921
LBL+RBL(CO)-CWS	Control mice	2.751	2.509	1.454	0	0.989
LBL+RBL(F)-FWB	Control mice	1.332	1.706	1.603	0	1.640
LBL+RBL(F)-FWS	Control mice	1.738	0	1.371	0	0

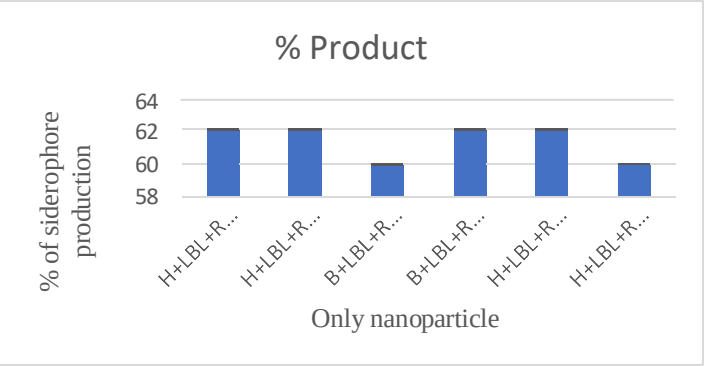
TABLE 4.4-The antibiotic zone of inhibition table

4.5 Siderophore results









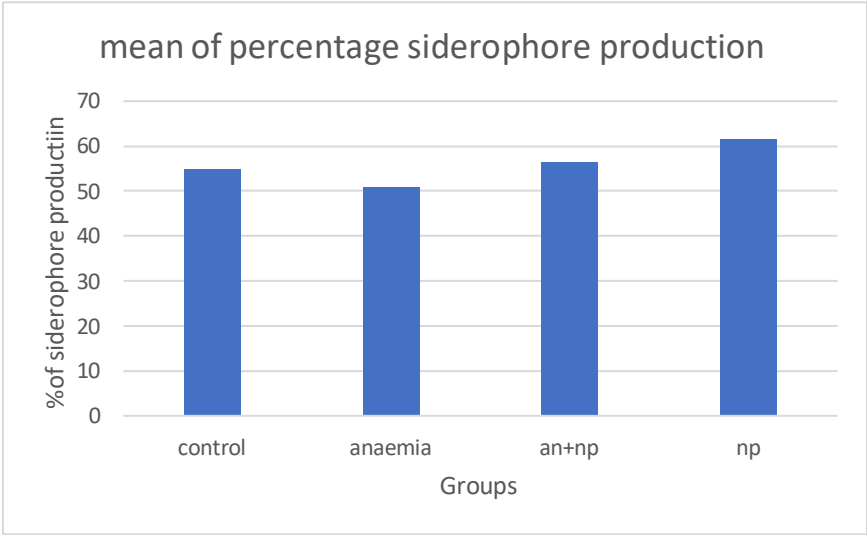
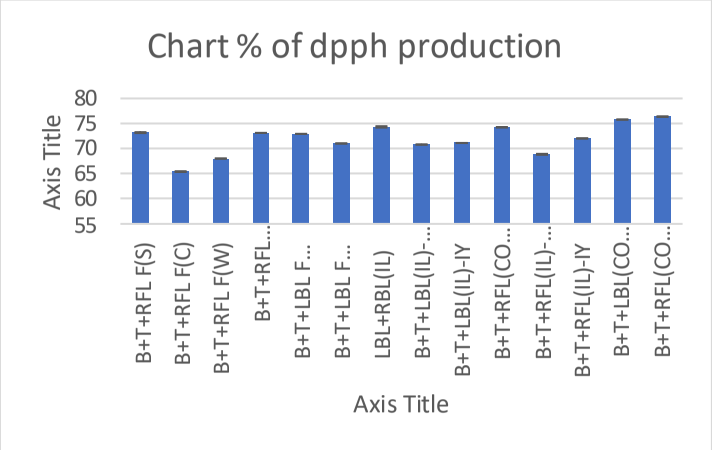
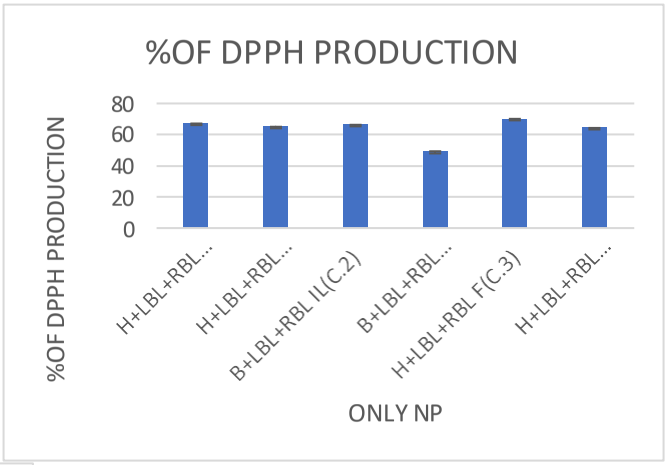
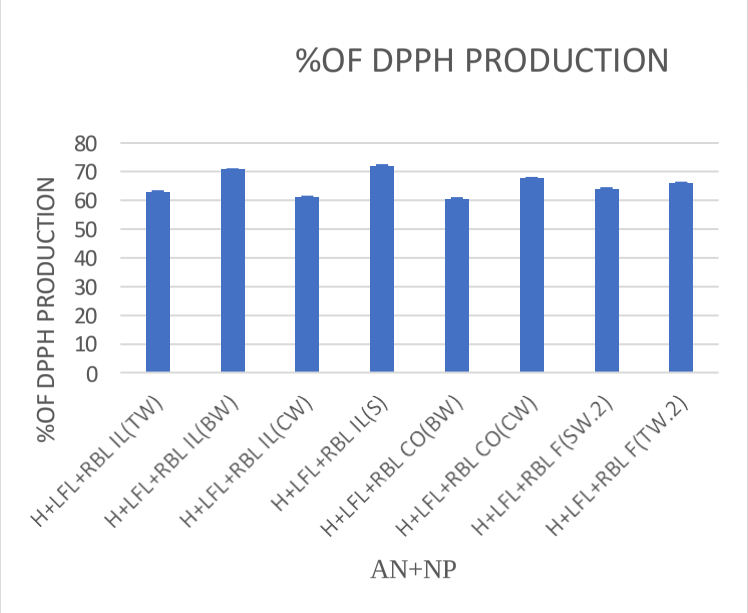


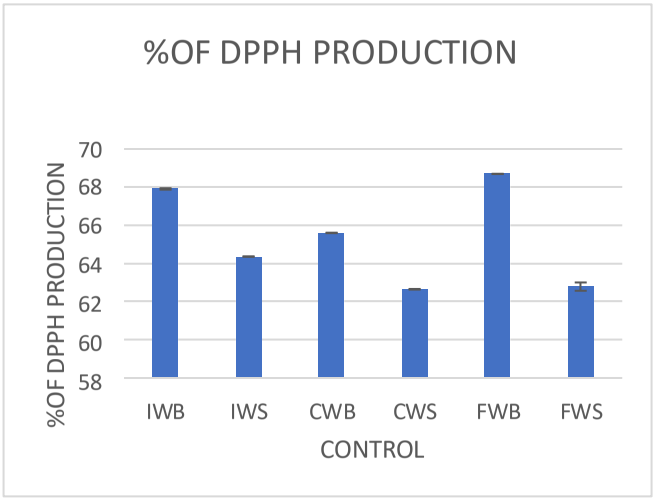
Fig4.5.2- the Graph of the siderophore assay of the individual strains as well as the groups

4.6- DPPH RADICAL SCAVENGING ASSAY









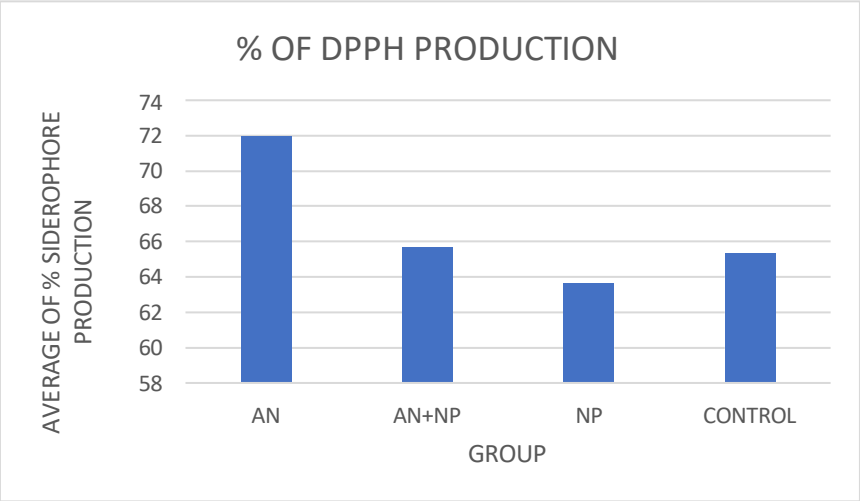
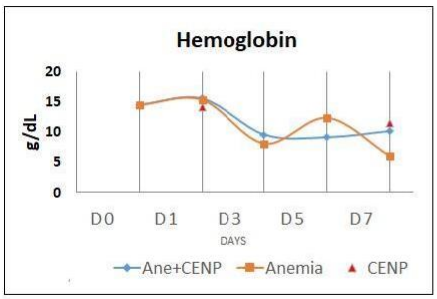
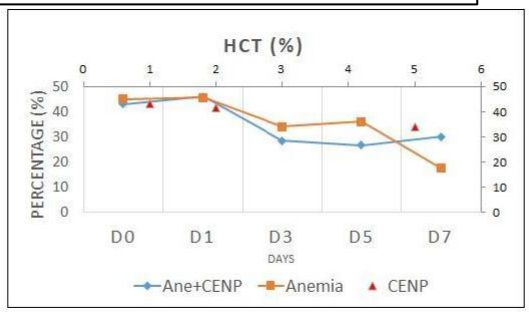


Fig 4.6.2- the Graph of the DPPH radical scavenging assay of the individual strains as well as the groups

4.7 -MacConkey agar plate

Fig-4.7-The result of the MacConkey agar plate with *E. coli* as a control





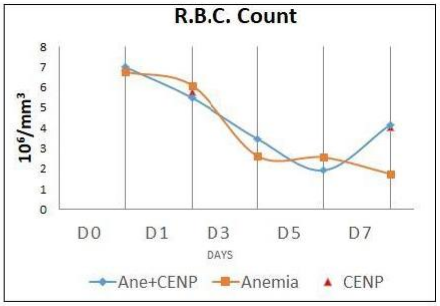


Fig-4.9 -The graph of the RBC cell count, CT and Hemoglobin

4.8 Histology pictures

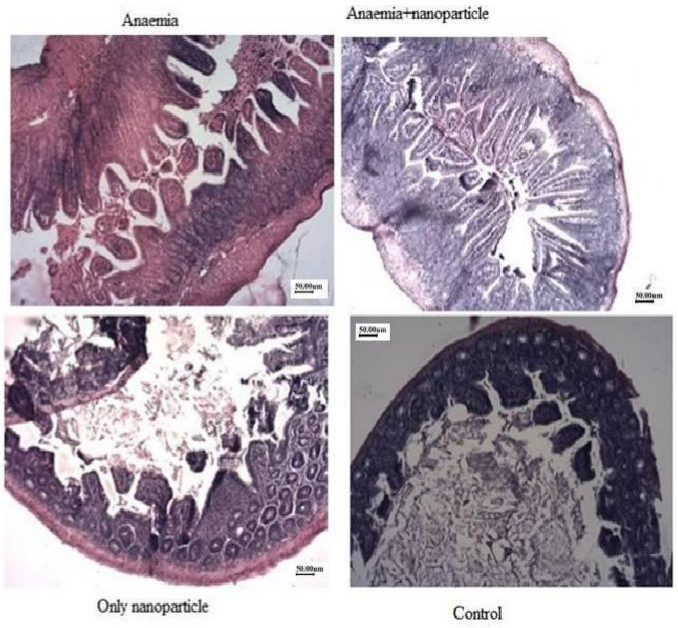


FIG-4.8.1-Ileum 100X

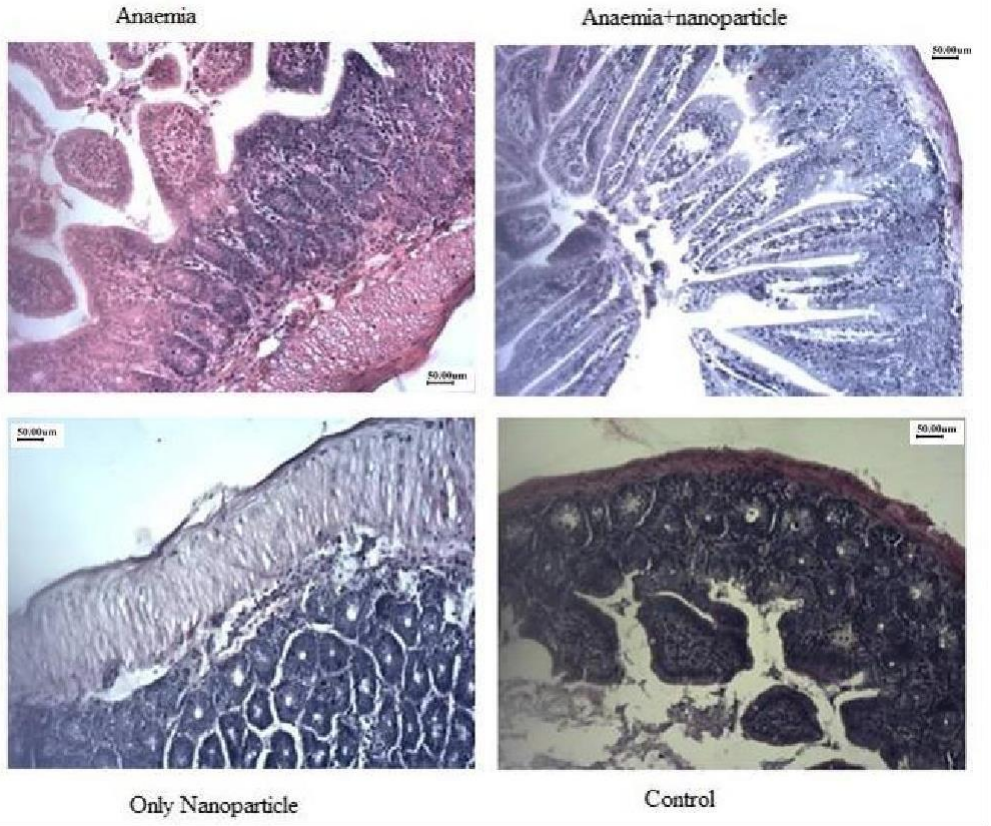


FIG-4.8.2-Ileum 200X

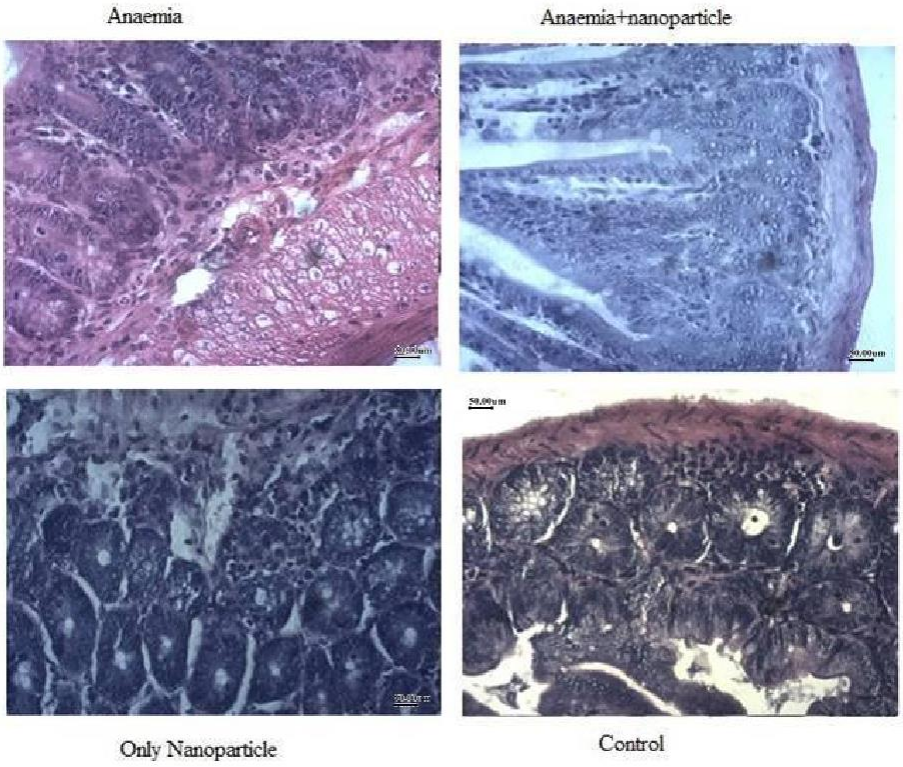


FIG-4.8.3-Ileum 400X

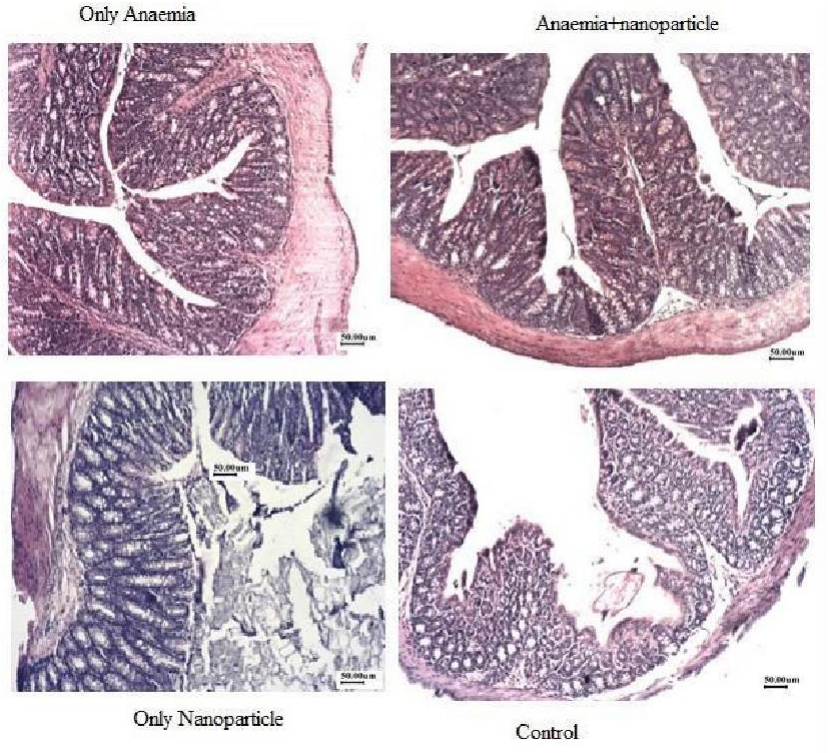


FIG-4.8.4-COLON 100X

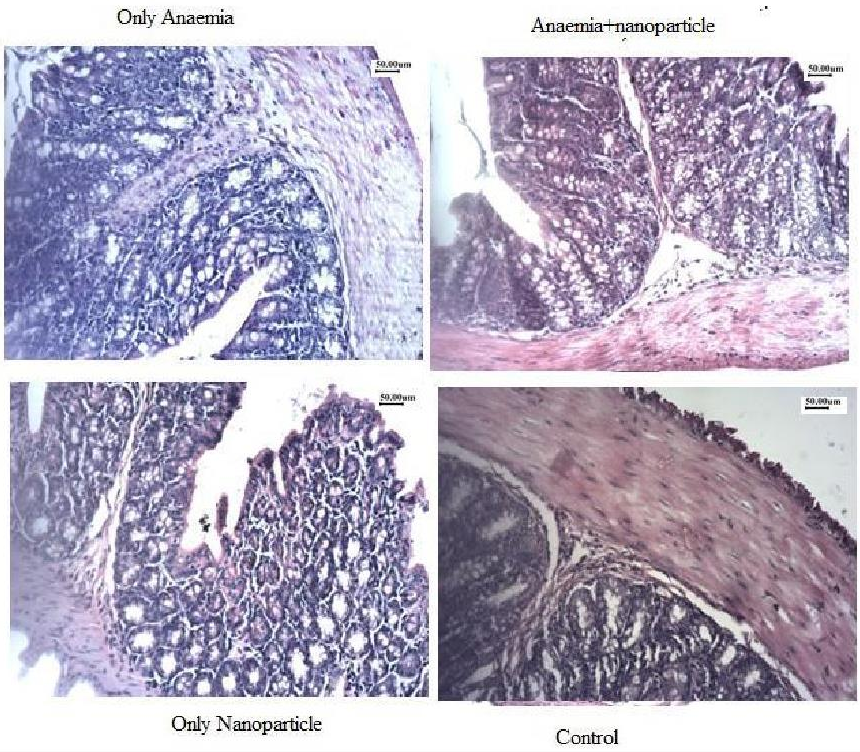


FIG-4.8.5- COLON 200X

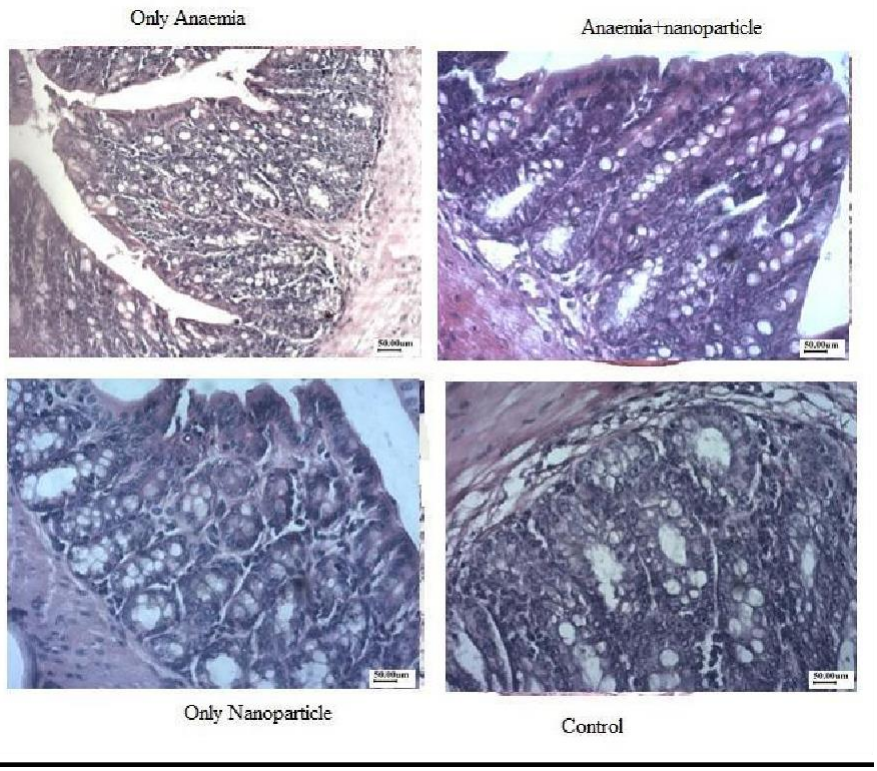


FIG-4.8.6- COLON 400X

5. DISCUSSION

This study was aimed to investigate the effect of green synthesized iron nanoparticles (FeNPs) by *Colocasia esculenta* leaf extract on gut microbiota of phenyl hydrazine-induced acute anaemic Swiss albino mice. The aim was beyond microbial enumeration and directed to functional profiling through biochemical tests, antibiotic resistance, antioxidant and siderophore production, and histopathological examination. These systems practice has been informative in our studies of how nanotherapeutics interact with microbial homeostasis in disease models.

Model Design and Microbiota Isolation

The anaemia model was induced successfully by injecting phenyl hydrazine (PhZ) intravenously, and the administered compound was known to cause oxidative damage in RBCs. Model groups were assigned into: (i) control (healthy), (ii) anaemia only, (iii) anaemia + FeNP and (iv) FeNP-only mice. The ileum, colon, and faecal content were employed to the microbial isolation by the serial dilution and nutrient agar spread plating method. Pure colonies were isolated by plating the mixed colonies, and the colony characteristics, such as shape, color, and elevation, which varied between groups on the plates, showed the diversity in the bacterial populations.

MacConkey Agar and Differentiation

Selective and differential MacConkey agar test showed that some, mostly strains from the anaemia + FeNP and control groups, were lactose-fermenting strains, with production of acid evident by red and pink colonies. This suggests that Gram-negative enteric, i.e., *E. coli*, may be benefiting from enhanced iron availability arising from FeNP treatment. In comparison, some strains in the anaemia only group exhibited low or no lactose fermentation, suggesting a shift away from fermentative bacteria in diseased conditions.

The dominant part of the gut microbiota of the anaemic group was Gram-positive cocci and bacilli, together with some Gram-negative bacilli. The effective rate of FeNP treatment was broader spectrum like GNR which was relevance with recovery of microbial flora. In contrast, in control and FeNP only groups Gram-positive cocci were predominant indicating eubiosis.

IMViC series provides a distinction among *Enterobacteriaceae* members:

Indole was more commonly produced in FeNP treated and NP only groups as compared to the anaemic mice, suggesting an augmentation of tryptophan metabolism. Methyl red (MR) positive test was observed in the respective groups which indicate mixed acid fermentation tests were selectively positive, particularly in FeNP-only groups, indicating acetoin production, and consequently, *Enterobacter spp*. Citrate utilization and urease activity were also higher in nanoparticle exposed groups, indicative of what may be construed as a more original lifestyle probably induced by improved iron availability. (Triple Sugar Iron) Tests-The slant and butt color change together showed glucose and lactose/sucrose fermentation. For the most part, anaemic clones showed a reduced capacity for sugar utilisation. Catalase-positive strains were numerous in FeNP and control groups, whereas coagulase-positive strains were less common and were mainly observed in anaemic mice, indicating that possible 'opportunistic' pathogens including S. aureus were present during the disease. This test has shown a marked dissimilarity in antibiotic resistance profile: Anaemic mice strains showed broad resistance,(which were ampicillin and methicillin, respectively), suggesting higher susceptibility to multi-drug-

resistant infections when challenged in an iron-starved state. The FeNP-induced groups were moderately to highly sensitive, predominantly to tetracycline and chloramphenicol (inhibiting zones ranged from 1.3 to 3.3 cm).Bacteria from the FeNP-only group exhibited lower resistance, suggesting that the microbiome was benefiting from a low pathogenic load. Vancomycin was moderately effective in all groups. The enhanced antibiotic sensitivity in NP-.treated groups suggests a role of FeNP in suppressing resistant pathobionts and promoting commensal microbiota

Siderophore producing capacity was quantified by spectrophotometrically method using CAS colorimetric assay. Siderophore activity was detected in all strains and with: Maximal production was seen in anaemic mice, demonstrating an iron-deprived gut environment inducing expression of siderophore genes. Intermediate levels in FeNP-treated groups, indicating that the restoration of iron levels decreases microbial competition. The least production was observed in the FeNP-only group, indicating sufficient iron availability. The results are consistent with the hypothesis that nanoparticle-mediated iron supplementation interferes with bacterial iron acquisition systems, thus reducing the predominance of siderophore-expressing pathogens such as.*Pseudomonas* and *E. coli*.

Antioxidant potential of bacterial metabolites was determined using DPPH assay: All strains showed radical scavenging effect; the maximal effect was detected with the strain from the FeNP-only group and FeNP-treated anaemic group. Anaemic lines were less able to neutralize free radicals suggesting greater gut oxidative stress. The high antioxidant activity in FeNP-treated groups indicates balancing of the redox homeostasis perhaps owing to the role of iron in enzymatic antioxidant pathways such as catalase and peroxidase.
This information supports the systemic advantages of FeNPs not just in microbial homeostasis but also in oxidative stress reduction.
The histology of the ileum and colon was as follows in various groups:
Anaemic mice: Mucosal erosion, crypt crypts shortening and inflammatory cellular infiltration.
FeNP group: Mucosal damage amelioration, villi restoration, and normalized crypts architecture.
FeNP-only: The mucosa was normal with intact epithelial lining and regular goblet cell distribution.
Control: Normal healthy morphology was seen. This finding supported the protective potential of FeNPs in reduction of tissue injury caused by anaemia, probably by re-establishment of microbial equilibrium and redox balance.

The haemoglobin level dropped precipitously in the anemia group (PHZ-treated) from 15.6 g/dL on Day 1 to 6.3 g/dL on Day 7. This significant reduction is compatible with Phenylhydrazine haemolytic anemia that generates oxidative damage in erythrocytes, transforming haemoglobin to methaemoglobin and causing RBC lysis. This means that the anaemic model was successfully created. In contrast, on the other side the NP+AN group showed initial decline to haemoglobin content(to ~8.3 g/dL by Day 3), but was soon recovered by Day 7 (~10.5 g/dL).
The CENP only group showed stable haemoglobin content throughout the process(14.5–15.8 g/dL), reflecting no haemolytic impact from the nanoparticle administration.

Haematocrit values also declined significantly in the anemia group, from ~47% on Day 1 to ~22% by Day 7. This is what was seen on haemoglobin level and can be said that haemolytic anaemia was successfully induced. In the anemia + CeNP group, HCT dropped initially but showed a partial restoration to ~32% by Day 7. The CeNP-only group maintained stable HCT values (around 43–46%), suggesting a lack of erythrocyte destruction and overall hematological stability RBC count also showed the same as it decreased significantly in

Anaemic groups. For CENP first there was a decrease in count but was later restored due to the CENP nanoparticle. For only CENP it maintained a consistent RBC count.

The anaemic environment caused the strains to form biofilm to survive the harsh, stressful conditions in the gut. They showed strong biofilm formation compared to control healthy mice where there is no biofilm formation. Firstly, the iron-deficient state of the anaemic host may impose selective pressure for the survival of stress-resistant and opportunistic bacteria that characteristically form biofilms to withstand hostile environments. Conversely, the control strains from healthy, non-anaemic mice showed poor or no biofilm production. This indicates that the control has a non-pathogenic and balanced gut microbiota that can be composed of commensal bacteria that do not require biofilm production for their survival due to stable gut conditions.

6. CONCLUSION

This study offers a valuable way to deepen the understanding of the complex interplay between iron availability, nano-particle based iron supplementation and the gut microbial ecology via an anaemic murine model. Feasibility of green-synthesized iron nanoparticles from Colocasia esculenta in controlling iron-deficiency anaemia and their hypothetical mechanisms of action on the system and microbes was reported for the first time. The study integrated various analytical tools such as biochemical analysis, histological observation, antibiotic susceptibility test, antioxidant screening and siderophore detection to evaluate the physiological and microbiological features in response to the intervention. The scope of this investigation has provided a compelling body of evidence that green iron nanoparticles do not just return the systemic iron status to baseline but that they contribute significantly to the recovery of gut microbial homeostasis and structure

Iron is essential for a multitude of physiological processes such as oxygen transport, energy metabolism, and DNA replication. Nevertheless, the high or low iron condition becomes a major problem in the clinical and microbiological field. Iron deficiency anaemia, particularly in low-resource settings, is a frequently under-diagnosed and inadequately managed condition. Herein, we aim to establish a connection between sustainable nanoparticle technology and medical drug delivery using the environment-friendly counterpart which is biocompatible as well—iron nanoparticles synthesized using Colocasia esculenta, otherwise known to possess antioxidation and medicinal properties.

Treatment of phenyl hydrazine-induced anaemia in mice led to a significant gut dysbiosis, expressed by changes in the bacterial composition, assessed by selective growth on MacConkey agar (Supple table 1) and confirmed by Gram staining. These modifications together with reduced sugar fermenting abilities (TSI) and increased siderophore production, indicate a microbiota subject to nutritional and oxidative pressure. Loss of commensal bacteria or the emergence of antibiotic-resistant strains also observed in the Kirby-Bauer assay, further highlights the subtle balance of microbial homeostasis perturbed by iron.

The increasing frequency of catalase-negative urease-positive bacteria in the anaemic mice also suggests a skew towards opportunistically pathogenic colonization. The FeNPs induced marked attenuation of the changes. This data indicates enhanced fermentation, increased metabolic versatility (IMViC and citrate employment assays) and decreased siderophore production indicative of improved iron availability in the gut. Once again, the higher DPPH radical scavenging activity in the nanoparticle-treated groups also confirmed that FeNPs not only reduce free radical generation but also support the colonization of beneficial antioxidant-producing microbes

One of the most obvious findings was the histological recovery of the gut tissues of nanoparticle-fed mice. Reversion of crypt damage, villi shortening, and mucosal inflammation suggests that FeNPs has more than a micronutrient effect to play a therapeutic role. This may further support epithelial regeneration and barrier integrity, possibly through the modulation of microbial metabolites, such as SCFA, known to affect gut architecture." Nanoparticle only-group did not present any histopathological abnormalities and confirmed the biocompatibility and safety of green-synthesized FeNPs.

Further, antibiotic susceptibility profiles indicated a reduction in resistance among the nanoparticle-exposed group, thus, suggesting a potentiality of FeNPs in indirectly addressing Antimicrobial Resistance (AMR). Restoration of iron could counteract the survival advantage of pathogenic bacteria that produce siderophores, hence altering the competitive microbial landscape and lowering the burden of AMR.

The present work adds new knowledge to the nascent field of nanomedicine, A novel demonstration of how iron particles, in the presence of disease, could alter the gut microbial ecology. In contrast to typical oral iron formulations that can worsen gut dysbiosis or inflammation, spectral FeNPs made in a green manner appeared to improve systemic and microbial health. In addition, the environmentally friendly Colocasia esculenta-based synthesis offers solutions to the toxic health concerns linked to chemically synthesized nanoparticles.

In addition, the implications of this study could have a broader effect on clinical nutrition, microbiology, environmental sustainability, and public health. If the same can be achieved in human medicine, it might transform the treatment of iron-deficiency anaemia, particularly in those populations who already have an imbalance in their gut microbiota, either because they are malnourished or because there's been overuse of antibiotics in poor-regions of the world.

7. FUTURE PROSPECTS

The results of this investigation will provide a positive springboard for further research in the interconnection of nanomedicine, gut microbiology, and nutritional intervention Although the current study affirms the potential use of green synthesized iron nanoparticles (FeNPs) to modify gut microbiota, alleviate oxidative stress and normalize integrity of intestine in anaemic mice, however, the present the current study leaves a lot of space for further exploration. These opportunities are essential to implement preclinical discoveries into viable, safe, and effective clinical therapies.

In future work, NGS, including 16S rRNA and whole genome metagenomic, analysis should be used to further investigate taxonomic and functional variations within the gut microbiota following FeNP post-treatment. This could enable accurate determination of beneficial and destructive bacterial genera and species, such as *Lactobacillus*, *Bifidobacterium*, and *Escherichia coli*. Also, functional genomic techniques may reveal FeNP effects on microbial genes involved in siderophore production, oxidative stress resistance and metabolic versatility. Because of the impact of gut microbiota on iron absorption and general gut health, nutraceutical associations of FeNPs with probiotics or prebiotics might offer a beneficial synergy. Co-deposition with lactobacilli such as Lactobacillus plantarum or fibres i.e. inulin may increase colonization of these beneficial microbes, reduce gastrointestinal side effects and enhance iron bio accessibility. Studying these combinations may allow the development of more effective, microbiome-sparing treatment regimens.

In order to clarify the special advantages and disadvantages of iron nanoparticles, comparative study with other metal nanoparticles including zinc, silver, and copper is needed. These comparisons could be critical for the discovery of nanoparticle specific effects on the microbiota composition, tissue interactions and health benefit and will provide essential information in the design of multi-metal nanoparticle therapeutics for complex diseases.

To summarize, the promising future of FeNP-based therapy is interdisciplinary cross application in the fields of nanotechnology, microbiology, pharmacology, and clinical science. With their enhanced safety, specificity, and sustainable production attention, green synthesized FeNPs have the potential to completely redefine not only iron supplementation, but the gut health therapeutics in general.

8. APPENDIX

FIG 8.1-The DPPH results of the bacterial strains with control

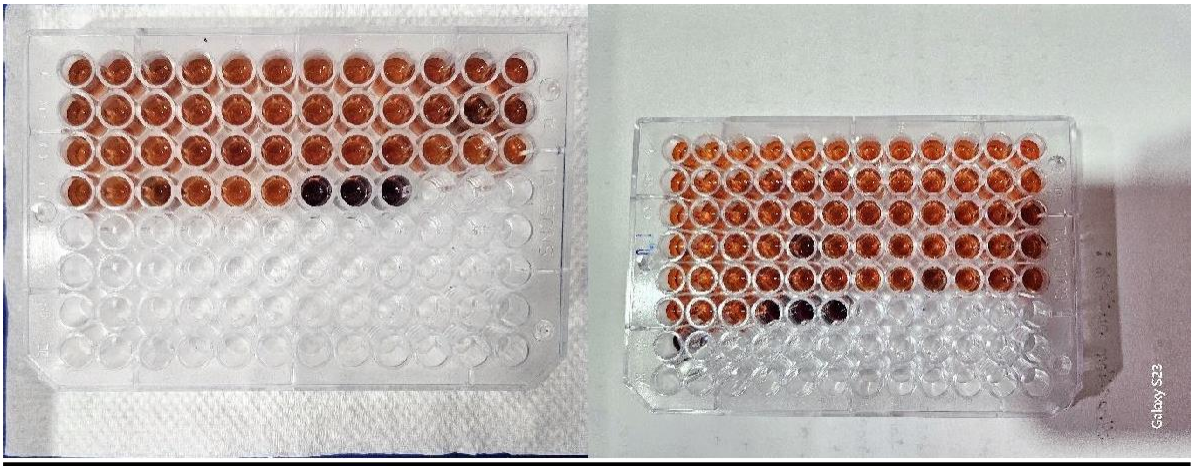


FIG 8.2-The siderophore results of the bacterial strains with control

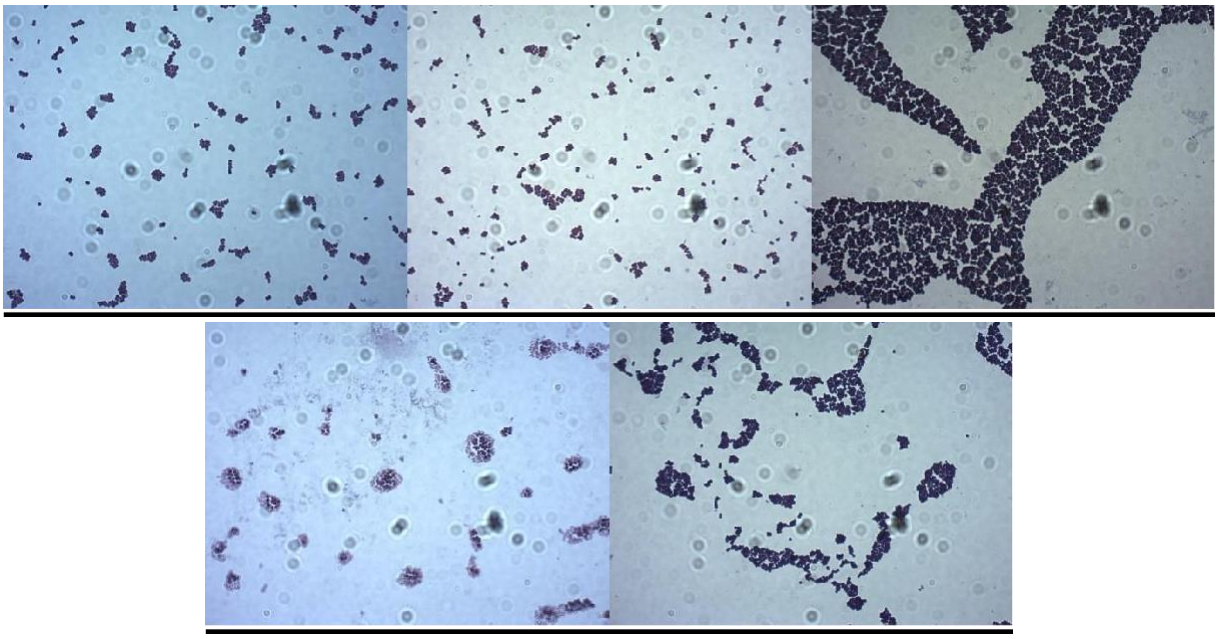


Fig 8.3- Some gram staining pictures comprising Gram positive coccus and Gram-negative coccus

FIG-8.4- Some citrates and urease test results of the remaining strains

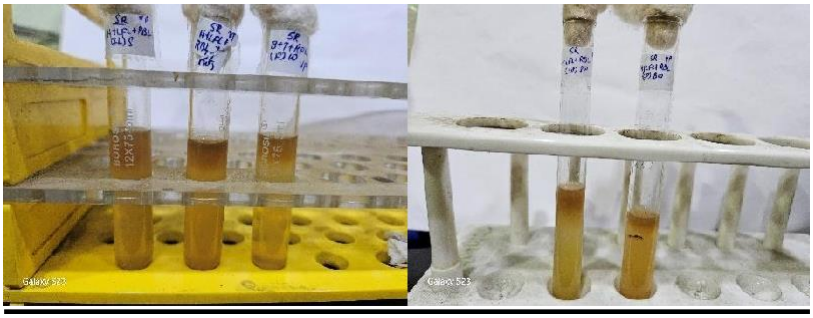


FIG-8.5- Some indole, MR and VP test results of the remaining strains

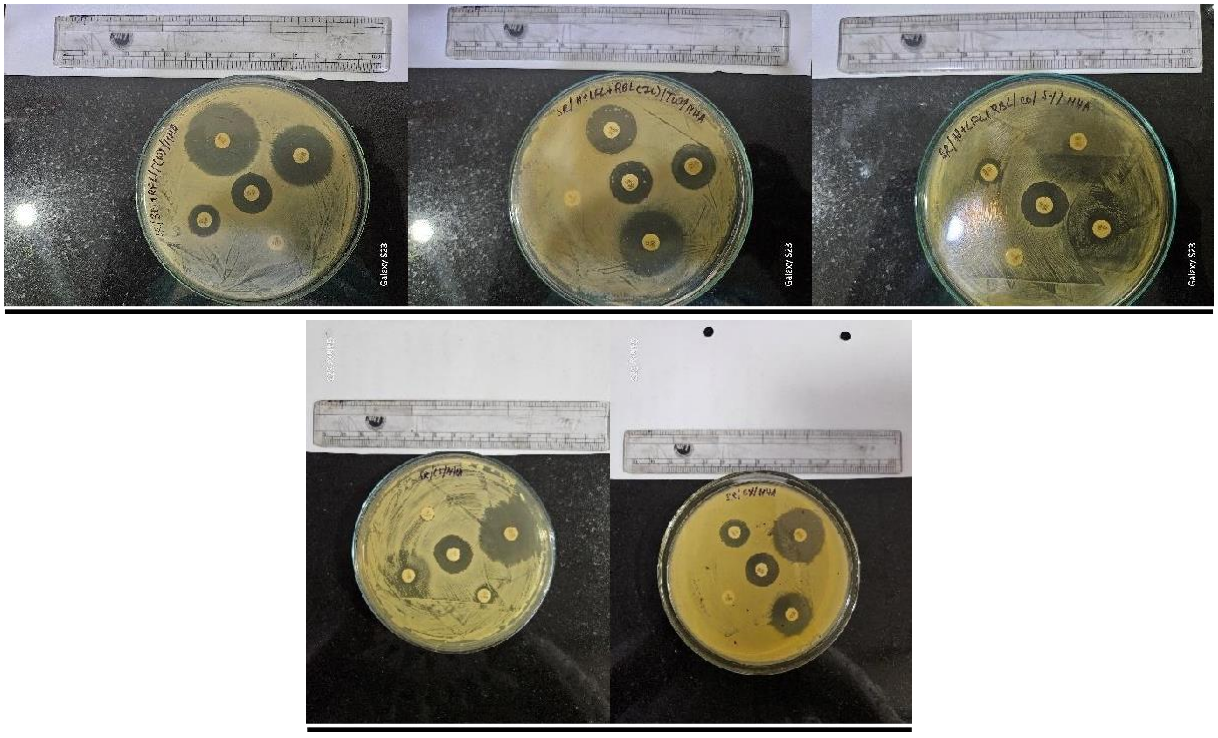


FIG-8.6- Some antibiotic zone of inhibition pictures of the remaining strains

FIG-8.7- Some catalase and coagulase pictures of the remaining strains

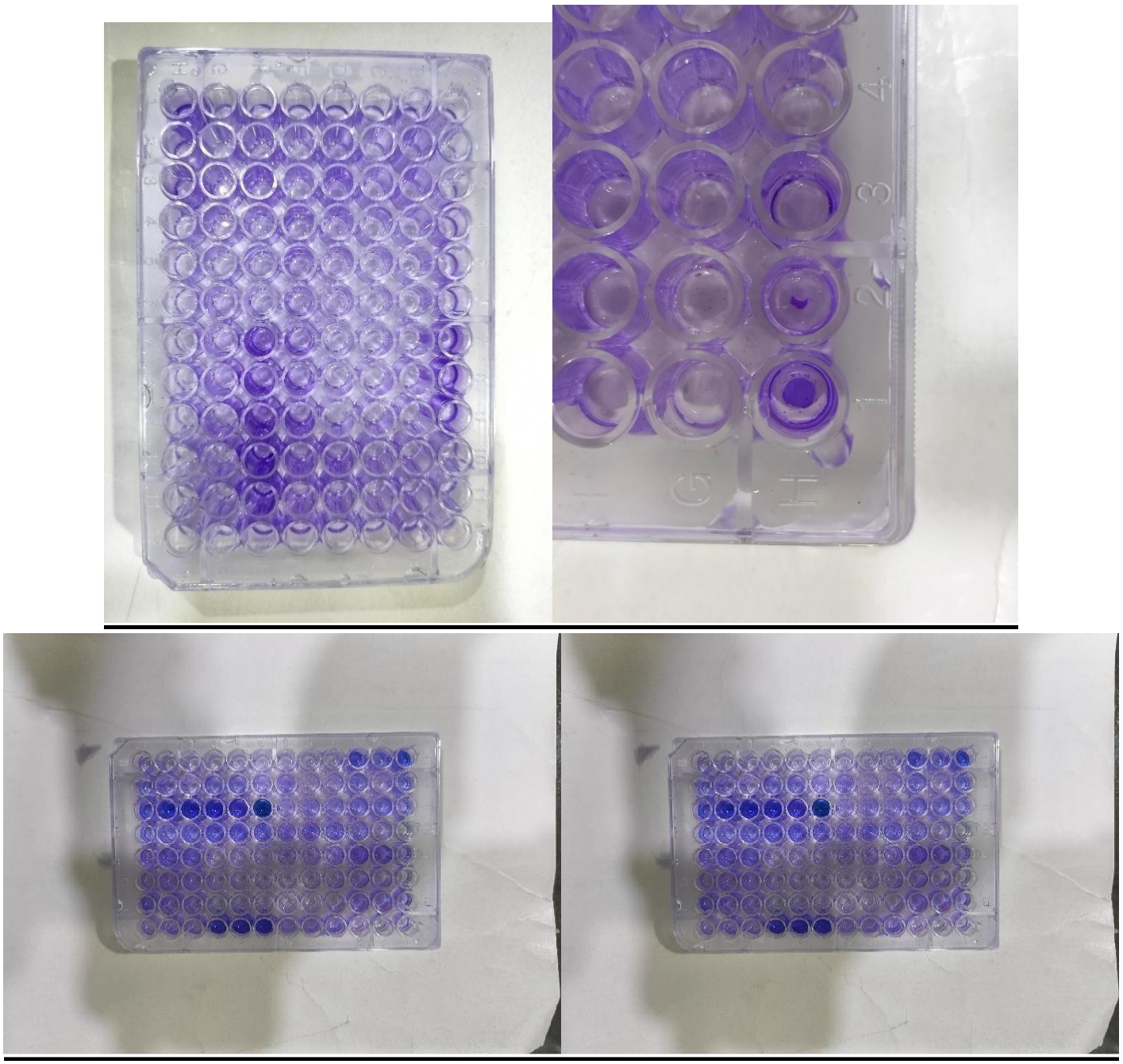


FIG-8.7- The biofilm formation pictures on the strains of Only anemia and control

LIST OF FIGURES

SERIAL NO	FIGURE NUMBER	FIGURE NAME
1.	FIG-4.1.1	Spread plate pictures of only nanoparticle
2.	FIG-4.1.2	Spread plate pictures of - Anaemia+nanoparticle
3.	FIG-4.1.3	Spread plate pictures of Only anaemia
4.	FIG-4.1.4	Spread plate pictures of Control
5.	FIG-4.2.1	The pure colony isolation plates of the animals
6.	FIG-4.3.1	Gram - bacillus, gram positive coccus, gram - rods and gram - coccus is found in anaemic mice
7.	FIG 4.3.2	Gram positive coccus, gram - rods and gram-negative coccus is found in anaemic+nanoparticle mice
8.	FIG-4.3.3	Only gram-positive coccus is found in only nanoparticle mice
9.	FIG-4.3.4	Only gram positive and gram - coccus is found in only nanoparticle mice
10.	FIG-4.4.A.1.	The indole result for some of the strains with the control
11.	FIG - 4.4.B.1	The methyl red results with control
12.	FIG- 4.4.C.1	The Vogue Proskauer results with control
13.	FIG- 4.4.D.1	The citrate results with control.
14.	FIG - 4.4.E.1	TSI result with positive control
15.	FIG 4.4.F.1	The Urease result with control
16.	FIG 4.4.G.1	Catalase strains with control
17.	FIG 4.4.H.1	Result of coagulase with control
18.	FIG 4.4.1	Antibiotic susceptibility test for anaemic,anaemia+nanoparticle,only nanoparticle and control mice
19.	Fig4.5.2	the Graph of the siderophore assay of the individual strains as well as the groups
20.	Fig 4.6.2	the Graph of the DPPH radical scavenging assay of the individual

		strains as well as the groups
21.	Fig-4.7	The result of the MacConkey agar plate with E.coli as a control
22.	FIG-4.8.1	Histology pictures of Ileum 10X
23.	FIG-4.8.2	Histology pictures of Ileum 20X
24.	FIG-4.8.3	Histology pictures of Ileum 40X
25.	FIG 8.1	The DPPH results of the bacterial strains with control
26.	FIG 8.2	The siderophore results of the bacterial strains with control
27.	Fig 8.3	Some gram staining pictures comprising Gram positive coccus and Gram-negative coccus
28.	FIG-8.4	Some citrates and urease test results of the remaining strains
29.	FIG-8.5	Some indole, MR and VP test results of the remaining strains
30.	FIG-8.6	Some antibiotic zone of inhibition pictures of the remaining strains
31.	FIG-8.7	Some catalase and coagulase pictures of the remaining strains
32.	Fig-4.9 -	The graph of the RBC cell count, CT and Hemoglobin
33.	FIG-4.8.4	Histology pictures of COLON 10X
34.	FIG-4.8.5	Histology pictures of COLON 20X
35.	FIG-4.8.6	Histology pictures of COLON 40X
36.	FIG-8.7	The biofilm formation pictures on the strains of Only anemia and control

LIST OF TABLES

SERIAL NO	FIGURE NUMBER	FIGURE NAME
1.	Table 1	Impact of oral iron supplementation on gut microbiota composition (decrease and increase)
2.	TABLE 4.4.1	The results of the IMViC tests
3.	TABLE 4.4.2	The citrate ,urease and tsi test respectively
4.	TABLE 4.4	The antibiotic zone of inhibition table

5.	Table -4.1	COLONY MORPHOLOGY
6.	Table 4.2	GRAM STAINING RESULTS AND PICTURES
7.	TABLE 4.4.1	The results of the IMViC tests

REFERENCES

1. Adak A, Khan MR. An insight into gut microbiota and its functionalities. Vol. 76, Cellular and Molecular Life Sciences. Birkhauser Verlag AG; 2019. p. 473–93.

2. Cappellini MD, Musallam KM, Taher AT. Iron deficiency anaemia revisited. Vol. 287, Journal of Internal Medicine. Blackwell Publishing Ltd; 2020. p. 153–70.

3. Stelle I, Kalea AZ, Pereira DIA. Symposium 1: Competition and bioavailability of dietary components: Iron deficiency anaemia: Experiences and challenges. In: Proceedings of the Nutrition Society. Cambridge University Press; 2019. p. 19–26.

4. Levi M, Simonetti M, Marconi E, Brignoli O, Cancian M, Masotti A, et al. Gender differences in determinants of iron-deficiency anemia: a population-based study conducted in four European countries. Ann Hematol. 2019 Jul 1;98(7):1573–82.

5. Mahadea D, Adamczewska E, Ratajczak AE, Rychter AM, Zawada A, Eder P, et al. Iron deficiency anemia in inflammatory bowel diseases—a narrative review. Vol. 13, Nutrients. MDPI; 2021.

6. Ogbe RJ, Adoga GI, Abu AH. Antianaemic potentials of some plant extracts on phenyl hydrazine-induced anaemia in rabbits. Journal of Medicinal Plants Research [Internet]. 2010;4(8):680–4. Available from: http://www.academicjournals.org/JMPR

7. Berger J. Phenylhydrazine haematotoxicity. J Appl Biomed. 2007;5:125–30.

8. Mehmood U, Yasmeen S, Nazar R, Tiwari SK. Metallic Nanoparticles: Status and Prospect. In: Nanoparticles Reinforced Metal Nanocomposites: Mechanical Performance and Durability. Springer Nature; 2023. p. 127–59.

9. Relationship Between Structure And Antimicrobial Activity Of Zinc Oxide Nanoparticles_ An Overview.

10. Bogdanović U, Lazić V, Vodnik V, Budimir M, Marković Z, Dimitrijević S. Copper nanoparticles with high antimicrobial activity. Mater Lett. 2014 Aug 1;128:75–8.

11. Wang L, Hu C, Shao L. The antimicrobial activity of nanoparticles: Present situation and prospects for the future. Vol. 12, International Journal of Nanomedicine. Dove Medical Press Ltd.; 2017. p. 1227–49.

12. Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, et al. Antimicrobial effects of silver nanoparticles. Nanomedicine. 2007 Mar;3(1):95–101.

13. Palza H. Antimicrobial polymers with metal nanoparticles. Vol. 16, International Journal of Molecular Sciences. MDPI AG; 2015. p. 2099–116.

14. Crisan MC, Teodora M, Lucian M. Copper nanoparticles: Synthesis and characterization, physiology, toxicity and antimicrobial applications. Vol. 12, Applied Sciences (Switzerland). MDPI; 2022.

15. Ren G, Hu D, Cheng EWC, Vargas-Reus MA, Reip P, Allaker RP. Characterisation of copper oxide nanoparticles for antimicrobial applications. Int J Antimicrob Agents. 2009 Jun;33(6):587–90.

16. Mohd Yusof H, Mohamad R, Zaidan UH, Abdul Rahman NA. Microbial synthesis of zinc oxide nanoparticles and their potential application as an antimicrobial agent and a feed supplement in animal industry: A review. Vol. 10, Journal of Animal Science and Biotechnology. BioMed Central Ltd.; 2019.

17. Durán N, Durán M, de Jesus MB, Seabra AB, Fávaro WJ, Nakazato G. Silver nanoparticles: A new view on mechanistic aspects on antimicrobial activity. Vol. 12, Nanomedicine: Nanotechnology, Biology, and Medicine. Elsevier Inc.; 2016. p. 789–99.

18. Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Vol. 27, Biotechnology Advances. 2009. p. 76–83.

19. Synthesis, characterization and evaluation of antimicrobial activity of zinc oxide nanoparticles.

20. Dakal TC, Kumar A, Majumdar RS, Yadav V. Mechanistic basis of antimicrobial actions of silver nanoparticles. Front Microbiol. 2016 Nov 16;7(NOV).

21. Paladini F, Pollini M. Antimicrobial silver nanoparticles for wound healing application: Progress and future trends. Vol. 12, Materials. MDPI AG; 2019.

22. Espitia PJP, Soares N de FF, Coimbra JS dos R, de Andrade NJ, Cruz RS, Medeiros EAA. Zinc Oxide Nanoparticles: Synthesis, Antimicrobial Activity and Food Packaging Applications. Vol. 5, Food and Bioprocess Technology. 2012. p. 1447–64.

23. Prabhu S, Poulose EK. Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. Int Nano Lett. 2012 Dec;2(1).

24. Mehravani B, Ribeiro AI, Zille A. Gold nanoparticles synthesis and antimicrobial effect on fibrous materials. Vol. 11, Nanomaterials. MDPI AG; 2021.

25. Zawrah MF, Abd El-Moez SI. Zawrah and Sherein I. Abd El-Moez Antimicrobial Activities of Gold Nanoparticles against Major Foodborne Pathogens [Internet]. Vol. 8, Life Science Journal. 2011. Available from: http://www.lifesciencesite.comhttp://www.lifesciencesite.com.

26. Rai A, Prabhune A, Perry CC. Antibiotic mediated synthesis of gold nanoparticles with potent antimicrobial activity and their application in antimicrobial coatings. J Mater Chem. 2010 Aug 2;20(32):6789–98.

27. Tao C. Antimicrobial activity and toxicity of gold nanoparticles: research progress, challenges and prospects. Vol. 67, Letters in Applied Microbiology. John Wiley and Sons Inc; 2018. p. 537–43.

28. Schneider MGM, Martín MJ, Otarola J, Vakarelska E, Simeonov V, Lassalle V, et al. Biomedical Applications of Iron Oxide Nanoparticles: Current Insights Progress and Perspectives. Vol. 14, Pharmaceutics. MDPI; 2022.

29. Nair AB, Morsy MA, Shinu P, Kotta S, Chandrasekaran M, Tahir MA. Advances of Non-Iron Metal Nanoparticles in Biomedicine [Internet]. Vol. 24, J Pharm Pharm Sci (www.cspsCanada.org). 2021. Available from: www.cspsCanada.org

30. Lu CH, Hsiao JK. Diagnostic and therapeutic roles of iron oxide nanoparticles in biomedicine. Vol. 35, Tzu Chi Medical Journal. Wolters Kluwer Medknow Publications; 2023. p. 11–7.

31. Vallabani NVS, Singh S. Recent advances and future prospects of iron oxide nanoparticles in biomedicine and diagnostics. Vol. 8, 3 Biotech. Springer Verlag; 2018.

32. McNamara K, Tofail SAM. Nanoparticles in biomedical applications. Vol. 2, Advances in Physics: X. Taylor and Francis Ltd.; 2017. p. 54–88.

33. Banerjee R, Katsenovich Y, Lagos L, Mciintosh M, Zhang X, Li CZ. Nanomedicine: Magnetic Nanoparticles and their Biomedical Applications. Vol. 17, Current Medicinal Chemistry. 2010.

34. Pankhurst QA, Thanh NKT, Jones SK, Dobson J. Progress in applications of magnetic nanoparticles in biomedicine. J Phys D Appl Phys. 2009;42(22).

35. Magro M, Baratella D, Bonaiuto E, de A. Roger J, Vianello F. New Perspectives on Biomedical Applications of Iron Oxide Nanoparticles. Curr Med Chem. 2017 Jun 16;25(4):540–55.

36. Ittrich H, Peldschus K, Raabe N, Kaul M, Adam G. Superparamagnetic iron oxide nanoparticles in biomedicine: Applications and developments in diagnostics and therapy. RoFo Fortschritte auf dem Gebiet der Rontgenstrahlen und der Bildgebenden Verfahren. 2013 Dec 5;185(12):1149–66.

37. Soenen SJH, De Cuyper M. Assessing iron oxide nanoparticle toxicity in vitro: Current status and future prospects. Vol. 5, Nanomedicine. 2010. p. 1261–75.

38. Malhotra N, Lee JS, Liman RAD, Ruallo JMS, Villaflore OB, Ger TR, et al. Potential toxicity of iron oxide magnetic nanoparticles: A review. Vol. 25, Molecules. MDPI AG; 2020.

39. Liu G, Gao J, Ai H, Chen X. Applications and potential toxicity of magnetic iron oxide nanoparticles. Vol. 9, Small. 2013. p. 1533–45.

40. Paunovic J, Vucevic D, Radosavljevic T, Mandić-Rajčević S, Pantic I. Iron-based nanoparticles and their potential toxicity: Focus on oxidative stress and apoptosis. Vol. 316, Chemico-Biological Interactions. Elsevier Ireland Ltd; 2020.

41. Abakumov MA, Semkina AS, Skorikov AS, Vishnevskiy DA, Ivanova A V., Mironova E, et al. Toxicity of iron oxide nanoparticles: Size and coating effects. J Biochem Mol Toxicol. 2018 Dec 1;32(12).

42. Priya, Naveen, Kaur K, Sidhu AK. Green Synthesis: An Eco-friendly Route for the Synthesis of Iron Oxide Nanoparticles. Vol. 3, Frontiers in Nanotechnology. Frontiers Media S.A.; 2021.

43. KSV G. Green Synthesis of Iron Nanoparticles Using Green Tea leaves Extract. J Nanomedine Biotherapeutic Discov. 2017;07(01).

44. Herlekar M, Barve S, Kumar R. Plant-Mediated Green Synthesis of Iron Nanoparticles. Journal of Nanoparticles. 2014 Oct 2;2014:1–9.

45. Saif S, Tahir A, Chen Y. Green synthesis of iron nanoparticles and their environmental applications and implications. Vol. 6, Nanomaterials. MDPI AG; 2016.

46. Huang L, Weng X, Chen Z, Megharaj M, Naidu R. Green synthesis of iron nanoparticles by various tea extracts: Comparative study of the reactivity. Spectrochim Acta A Mol Biomol Spectrosc. 2014 Sep 15;130:295–301.

47. Arularasu M V., Devakumar J, Rajendran T V. An innovative approach for green synthesis of iron oxide nanoparticles: Characterization and its photocatalytic activity. Polyhedron. 2018 Dec 1;156:279–90.

48. Shahwan T, Abu Sirriah S, Nairat M, Boyaci E, Eroĝlu AE, Scott TB, et al. Green synthesis of iron nanoparticles and their application as a Fenton-like catalyst for the degradation of aqueous cationic and anionic dyes. Chemical Engineering Journal. 2011 Aug 1;172(1):258–66.

49. Pattanayak M, Nayak PL. ECOFRIENDLY GREEN SYNTHESIS OF IRON NANOPARTICLES FROM VARIOUS PLANTS AND SPICES EXTRACT [Internet]. Available from: www.ijpaes.com

50. Kaur M, Chopra DS. Glob J Nanomed Green Synthesis of Iron Nanoparticles for Biomedical Applications. Review Article. 2018;4.

51. Pandey K, Meena AK, Jain A, Singh RK. Molecular Mechanism of Phenylhydrazine Induced Haematotoxicity: A Review [Internet]. Available from: www.ajpct.org

52. Macfarlane S, Macfarlane GT. Composition and metabolic activities of bacterial biofilms colonizing food residues in the human gut. Appl Environ Microbiol. 2006 Sep;72(9):6204–11.

53. Buret AG, Motta JP, Allain T, Ferraz J, Wallace JL. Pathobiont release from dysbiotic gut microbiota biofilms in intestinal inflammatory diseases: A role for iron? 06 Biological Sciences 0605 Microbiology. J Biomed Sci. 2019 Jan 3;26(1).

54. Macfarlane S, Dillon JF. Microbial biofilms in the human gastrointestinal tract. In: Journal of Applied Microbiology. Blackwell Publishing Ltd; 2007. p. 1187–96.

55. Sheth RU, Li M, Jiang W, Sims PA, Leong KW, Wang HH. Spatial metagenomic characterization of microbial biogeography in the gut. Nat Biotechnol. 2019 Aug 1;37(8):877–83.

56. Fathi P, Wu S. Isolation, Detection, and Characterization of Enterotoxigenic Bacteroides fragilis in Clinical Samples. Open Microbiol J. 2016 Apr 18;10(1):57–63.

57. McClorry S, Zavaleta N, Llanos A, Casapía M, Lönnerdal B, Slupsky CM. Anemia in infancy is associated with alterations in systemic metabolism and microbial structure and function in a sex-specific manner: An observational study. American Journal of Clinical Nutrition. 2018 Dec 1;108(6):1238–48.

58. Long Y, Liang F, Guo R, Zhu C, Zhao X, Wang X, et al. Gut Microbiota Signatures in Gestational Anemia. Front Cell Infect Microbiol. 2021 Feb 25;11.

59. Donaldson GP, Lee SM, Mazmanian SK. Gut biogeography of the bacterial microbiota. Vol. 14, Nature Reviews Microbiology. Nature Publishing Group; 2015. p. 20–32.

60. Seyoum Y, Baye K, Humblot C. Iron homeostasis in host and gut bacteria–a complex interrelationship. Vol. 13, Gut Microbes. Bellwether Publishing, Ltd.; 2021. p. 1–19.

61. Long Y, Liang F, Guo R, Zhu C, Zhao X, Wang X, et al. Gut Microbiota Signatures in Gestational Anemia. Front Cell Infect Microbiol. 2021 Feb 25;11.

62. Earle KA, Billings G, Sigal M, Lichtman JS, Hansson GC, Elias JE, et al. Quantitative Imaging of Gut Microbiota Spatial Organization. Cell Host Microbe. 2015 Oct 14;18(4):478–88.

63. IRON NANOPARTICLE TREATMENT ON ANEMIA AND GUT MICROBES.

64. Arnoldini M, Cremer J, Hwa T. Bacterial growth, flow, and mixing shape human gut microbiota density and composition. Gut Microbes. 2018 Nov 2;9(6):559–66.

65. Soriano-Lerma A, García-Burgos M, Alférez MJM, Pérez-Carrasco V, Sanchez-Martin V, Linde-Rodríguez Á, et al. Gut microbiome–short-chain fatty acids interplay in the context of iron deficiency anaemia. Eur J Nutr. 2022 Feb 1;61(1):399–412.

66. Lamas B, Martins Breyner N, Houdeau E. Impacts of foodborne inorganic nanoparticles on the gut microbiota-immune axis: Potential consequences for host health. Vol. 17, Particle and Fibre Toxicology. BioMed Central Ltd.; 2020.

67. Campos D, Goméz-García R, Oliveira D, Madureira AR. Intake of nanoparticles and impact on gut microbiota: in vitro and animal models available for testing . Gut Microbiome. 2022;3.

68. Coyte KZ, Rakoff-Nahoum S. Understanding Competition and Cooperation within the Mammalian Gut Microbiome. Vol. 29, Current Biology. Cell Press; 2019. p. R538–44.

69. Muleviciene A, D'Amico F, Turroni S, Candela M, Jankauskiene A. Iron deficiency anemia-related gut microbiota dysbiosis in infants and young children: A pilot study. Acta Microbiol Immunol Hung. 2018 Dec 1;65(4):551–64.

70. Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. Vol. 69, Pharmacological Research. 2013. p. 52–60.

71. Lin L, Du Y, Song J, Wang W, Yang C. Imaging Commensal Microbiota and Pathogenic Bacteria in the Gut. Acc Chem Res. 2021 May 4;54(9):2076–87.

72. Ciont C, Mesaroș A, Pop OL, Vodnar DC. Iron oxide nanoparticles carried by probiotics for iron absorption: a systematic review. Vol. 21, Journal of Nanobiotechnology. BioMed Central Ltd; 2023.

73. von Martels JZH, Sadaghian Sadabad M, Bourgonje AR, Blokzijl T, Dijkstra G, Faber KN, et al. The role of gut microbiota in health and disease: In vitro modeling of host-microbe interactions at the aerobe-anaerobe interphase of the human gut. Vol. 44, Anaerobe. Academic Press; 2017. p. 3–12.

74. Bloor SR, Schutte R, Hobson AR. Oral iron supplementation—gastrointestinal side effects and the impact on the gut microbiota. Vol. 12, Microbiology Research. Page Press Publications; 2021. p. 491–502.

75. Shi J, Xie Y, Li Y, Ren D, Zhang Y, Shao H, et al. Effects of food-grade iron(III) oxide nanoparticles on cecal digesta- and mucosa-associated microbiota and short-chain fatty acids in rats. Biosci Microbiota Food Health. 2024;43(1):43–54.

76. Das A, Chaudhuri D, Ghate NB, Panja S, Chatterjee A, Mandal N. Protective effect of Clerodendrum colebrookianum leaves against iron-induced oxidative stress and hepatotoxicity in Swiss albino mice. Vol. 53, Indian Journal of Experimental Biology. 2015.

77. Shehzadi A, Bibi Z, Qadeer Sarwar M, Ullah A, Rehman A, Abbas Bukhari D. Effect of probiotics on hematological parameters of male and female Wistar rats. Saudi J Biol Sci. 2023 Oct 1;30(10).

78. Rusu IG, Suharoschi R, Vodnar DC, Pop CR, Socaci SA, Vulturar R, et al. Iron supplementation influence on the gut microbiota and probiotic intake effect in iron deficiency—A literature-based review. Vol. 12, Nutrients. MDPI AG; 2020. p. 1–17.

79. Gulcin İ, Alwasel SH. DPPH Radical Scavenging Assay. Vol. 11, Processes. Multidisciplinary Digital Publishing Institute (MDPI); 2023.

.

Mausumi Sikdar

Srijeeta thesis

Srijeeta review

FRG Review

Presidency University, Kolkata

Document Details

Submission ID

trn:oid:::1:3267905458

Submission Date

Jun 4, 2025, 12:17 AM GMT+5:30

Download Date

Jun 4, 2025, 12:45 AM GMT+5:30

File Name

final_thesis_submission_Srijeeta_Ghish_SNU.docx

File Size

4.7 MB

62 Pages

13,238 Words

74,318 Characters

3% Overall Similarity

The combined total of all matches, including overlapping sources, for each database.

Filtered from the Report

- Bibliography
- Quoted Text

Match Groups

- 8 Not Cited or Quoted 3%
  Matches with neither in-text citation nor quotation marks
- 0 Missing Quotations 0%
  Matches that are still very similar to source material
- 0 Missing Citation 0%
  Matches that have quotation marks, but no in-text citation
- 0 Cited and Quoted 0%
  Matches with in-text citation present, but no quotation marks

Top Sources

2% Internet sources
0% Publications
2% Submitted works (Student Papers)

Integrity Flags

1 Integrity Flag for Review

Replaced Characters
35 suspect characters on 11 pages
Letters are swapped with similar characters from another alphabet.

Our system's algorithms look deeply at a document for any inconsistencies that would set it apart from a normal submission. If we notice something strange, we flag it for you to review.

A Flag is not necessarily an indicator of a problem. However, we'd recommend you focus your attention there for further review.

Match Groups

- 8 Not Cited or Quoted 3%
  Matches with neither in-text citation nor quotation marks
- 0 Missing Quotations 0%
  Matches that are still very similar to source material
- 0 Missing Citation 0%
  Matches that have quotation marks, but no in-text citation
- 0 Cited and Quoted 0%
  Matches with in-text citation present, but no quotation marks

Top Sources

2% Internet sources
0% Publications
2% Submitted works (Student Papers)

Top Sources

The sources with the highest number of matches within the submission. Overlapping sources will not be displayed.

1	Student papers	
Presidency University, Kolkata		2%
2	Internet	
teresas.ac.in		<1%
3	Student papers	
Associatie K.U.Leuven		<1%